

" STUDIES ON THE INTERACTION OF CHOLESTEROL ANALOGUES  
WITH CHOLESTEROL OXIDISING SYSTEMS AND MEMBRANE  
" COMPONENTS.

IAIN F. CRAIG

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY  
IN THE UNIVERSITY OF EDINBURGH



## Acknowledgement

I should like to thank GSB, KES and all my friends in the laboratory for their help and encouragement.



## Abstract

A series of cholesterol analogues possessing side chains with either more or less carbon atoms than cholesterol were synthesised from hyodesoxycholic acid and fully characterised by nuclear magnetic resonance, mass spectrometry, and infra red analysis. These analogues were used in (a) physical studies of cholesterol phospholipid interactions and (b) experiments to investigate the substrate specificities of cholesterol 7 $\alpha$ -hydroxylase from rat liver and the adrenal mitochondrial cholesterol side chain cleavage enzyme.

(a) The physical properties of cholesterol and a series of cholesterol side chain analogues were investigated by incorporating them into liposomes prepared from lipid films containing the sterol and a series of saturated or unsaturated phospholipids in the required molar proportions. A series of spin labels (3-nitroxy octane, Tempo, 25 nitroxy cholestane, and 3 nitroxy cholestane) were incorporated into the sterol phospholipid liposomes and the effect of each analogue on each spin label mobility was observed. In addition the effect of each analogue on water permeability across the liposomal membrane was determined. The results of both sets of experiments showed that the cholesterol molecule with its iso-octane side chain was optimally adapted for maximal interaction with phospholipid. This specificity was not observed in monolayer studies of cholesterol phospholipid interactions.

(b) The involvement of the cholesterol side chain in cholesterol oxidation by the liver microsomal enzyme, cholesterol 7 $\alpha$ -hydroxylase was determined using an established assay based on the oxidation of added radioactive cholesterol. Using phenobarbitone pretreated rats it was shown that the metabolism of short side chain analogues was stimulated whereas 7 $\alpha$ -hydroxylase activity was unaffected. Thus it is postulated that the side chain of cholesterol determines its metabolic fate and that the shorter side chain analogues are metabolised by the

inducible hepatic drug metabolising enzyme system. A new G.L.C. assay which measures the production of  $7\alpha$ -hydroxy cholesterol from endogenous microsomal cholesterol has been developed and used to investigate the problem of substrate supply for the  $7\alpha$ -hydroxylase enzyme. This involved the addition of cholesterol in detergents in sonicated and unsonicated liposomes containing different molar proportions of sterol and in organic solvents to a cholesterol depleted form of the enzyme.

The involvement of a soluble protein isolated from the 100,000 x g supernatant of rat liver by ammonium sulphate fractionation, gel filtration and ion exchange chromatography has also been demonstrated. The molecular weight of the protein has been determined by S.D.S. polyacrylamide gel electrophoresis, and its means of action investigated.

The cholesterol side chain cleavage enzyme of rat adrenal mitochondria has been shown to possess an absolute requirement for cholesterol as substrate by use of a radio-immuno-assay that measures pregnenolone production from the exogenous cholesterol side chain analogues.

The results of both the physical measurements and the enzyme assays show that the side chain of cholesterol plays a major role in determining the extent of the interaction between cholesterol and phospholipids and the specificity of cholesterol protein interaction.

## CONTENTS

	<u>Page Number</u>
ABSTRACT	
<u>CHAPTER 1</u>	Introduction 1
<u>CHAPTER 2</u>	Materials and Methods 17
<u>CHAPTER 3</u>	Synthesis of Cholesterol Analogues with Altered Side Chains 33
<u>CHAPTER 4</u>	Spin Label Studies of Cholesterol Phospholipid Interactions 57
<u>CHAPTER 5</u>	The Use of Stopped Flow Spectro- photometry and Monolayers to Study the Interaction Between Cholesterol and Phospholipid 76
<u>CHAPTER 6</u>	Oxidative Metabolism of the Cholesterol Analogues by the Cholesterol Oxidation Enzyme Systems in Rat Liver Microsomes and Rat Adrenal Mitochondria 95
<u>CHAPTER 7</u>	Investigation of the Possible Involvement of a Supernatant Protein in Rat Liver Microsomal Cholesterol 7 $\alpha$ -Hydroxylase 139
<u>CHAPTER 9</u>	General Conclusion References 157

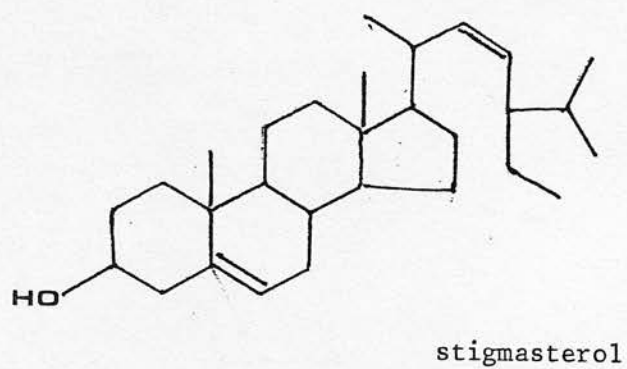
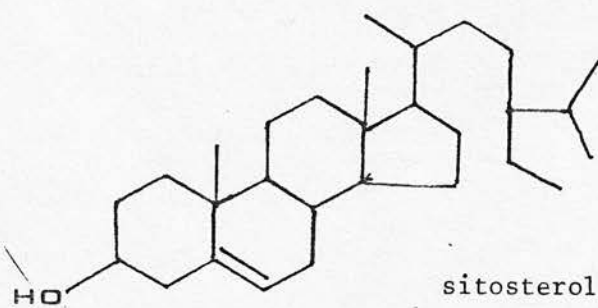
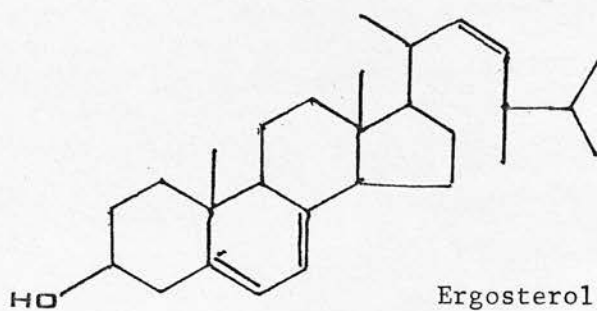
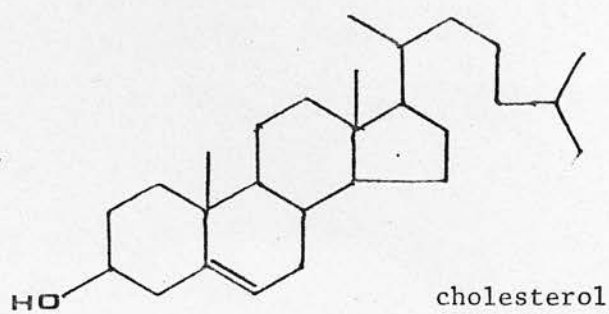
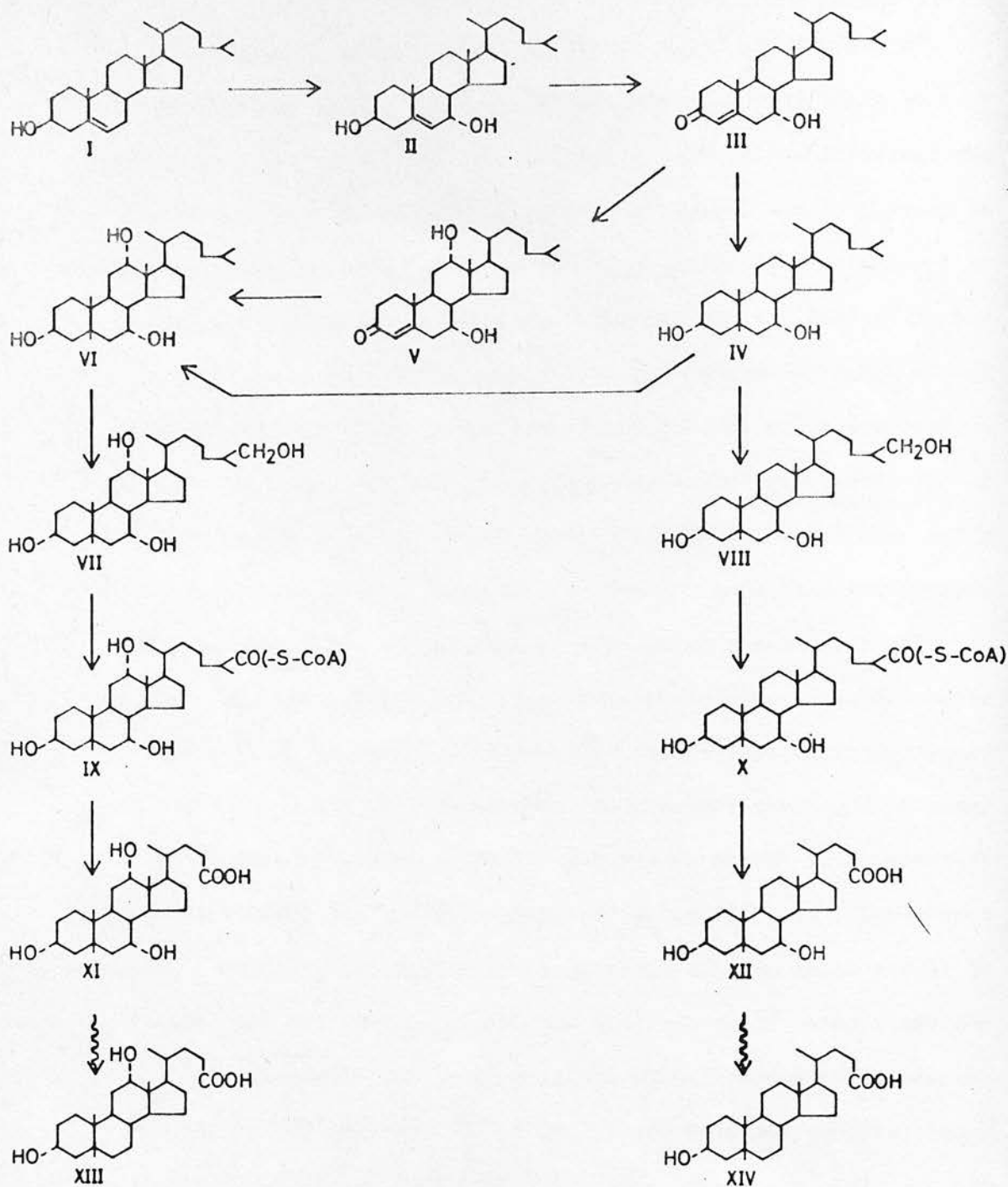


Fig. 1.1

## Introduction

Cholesterol is found in all mammalian cell membranes in varying quantities depending on the source. It is not present in any bacterial cells, but derivatives are present in plants and fungi of yeast. These sterols possess a double bond in the side chain at  $C_{22}$  and/or a methyl or ethyl group at  $C_{24}$  and include stigmasterol and sitosterol (plant) and ergosterol (fungi and yeast) (Fig. 1.1). In the mammalian system the cholesterol originates either from cholesterol newly synthesised in the animal or it may be obtained in the diet. In this introduction the dual role that cholesterol plays as a component of cellular membranes and as a substrate for cholesterol oxidising systems is considered.

The liver has a major role to play in the regulation of the amount of cholesterol that is present in animals. Dietary cholesterol, lipoprotein cholesterol and cholesterol synthesised in situ may increase the liver cholesterol content, whereas the excretion of cholesterol in faeces as neutral sterols such as cholestanol and coprostanol, the conversion of cholesterol to bile acid, the re-export of lipoproteins and the metabolism of cholesterol to steroid hormones and their catabolites decrease the liver content. Of the catabolic processes the conversion of cholesterol to the bile acids is quantitatively the most important and accounts for 50 per cent of the excreted cholesterol while the conversion to steroid hormones is of minor importance. The transformation of cholesterol to the bile acids requires many chemical modifications involving two hydroxylations of the sterol nucleus at the 7 and 12 positions, oxidation of the  $3\beta$  hydroxyl group, saturation of the  $C_5/C_6$  double bond and reduction of the 3 oxo group to a  $3\alpha$  hydroxyl. The side chain is reduced in length by a three carbon fragment by a series of hydroxylations and oxidations



**Figure 1.2** Pathways for formation of bile acids. I, cholesterol; II, 7 $\alpha$ -hydroxycholesterol (5-cholestene-3 $\beta$ , 7 $\alpha$ -diol); III, 7 $\alpha$ -hydroxy-4-cholesten-3-one; IV, 5 $\beta$ -cholestane-3 $\alpha$ , 7 $\alpha$ -diol; V, 7 $\alpha$ , 12 $\alpha$ -dihydroxy-4-cholesten-3-one; VI, 5 $\beta$ -cholestane-3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -triol; VII, 5 $\beta$ -cholestane-3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ , 26-tetrol; VIII, 5 $\beta$ -cholestane-3 $\alpha$ , 7 $\alpha$ , 26-triol; IX, 3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -trihydroxy-5 $\beta$ -cholestanoic acid; X, 3 $\alpha$ , 7 $\alpha$ -dihydroxy-5 $\beta$ -cholestanoic acid; XI, cholic acid; XII, chenodeoxycholic acid; XIII, deoxycholic acid; XIV, lithocholic acid.  $\rightarrow$ , reactions catalyzed by liver enzymes;  $\rightsquigarrow$ , reactions catalyzed by microbial enzymes.



(Fig. 1.2). The bile acids so formed are converted into their CoA derivatives in the liver and are condensed with glycine or taurine in the hepatocytes to give bile salts. Bile salts themselves are anionic detergents which emulsify fat in the duodenum prior to digestion by pancreatic lipase. Unlike the partially hydrolysed fats, the bile salts themselves are not absorbed by the proximal small intestine but pass onto the ileum. When absorption occurs they are recirculated in the portal blood to the liver and then to the bile for re-entry at the duodenum. This is known as enterohepatic circulation. The bile salts may also be further metabolised by bacteria in the intestinal tract to secondary bile acids which are also returned to the liver and rehydroxylated to primary bile acids. It is estimated that 90 per cent of the bile acids are reabsorbed in the region of the lower ileum.

The first step in the production of bile acids from cholesterol was deduced to be the  $7\alpha$ -hydroxylation of cholesterol (1). Cannulation of the bile duct or treatment with the anion exchange resin, cholestyramine, interrupts the enterohepatic circulation and increases the cholesterol  $7\alpha$ -hydroxylase activity 10 fold. Not only is this activity increased but the output of both cholic and chenodeoxycholic acid is increased. As the proposed pathways for the production of these two primary bile acids diverge after the  $7\alpha$  hydroxylation and no enzyme is stimulated by cholestyramine treatment nor is there any noticeable build up of intermediates in the pathway leading to bile acids, cholesterol  $7\alpha$ -hydroxylase is considered to be the first and rate limiting step in the conversion of cholesterol to bile acids.

The decrease in the bile acid concentration in the enterohepatic circulation caused by cholestyramine feeding precedes the rise in

Mitochondrial type electron transport chain.

NADPH  $\longrightarrow$  flavoprotein  $\longrightarrow$  ferredoxin  $\longrightarrow$  P450

Endoplasmic reticulum type electron transport chain.

NADPH  $\longrightarrow$  flavoprotein ( $\longrightarrow$  X)  $\longrightarrow$  P450

The electron transport chains involved in the  
oxidation of cholesterol by mitochondria and  
endoplasmic reticulum.

Fig. 1.3



cholesterol 7 $\alpha$ -hydroxylase activity. As puromycin, actinomycin D and cycloheximide all inhibit the rise in activity (2,3), most workers are agreed that the increase in cholesterol 7 $\alpha$  hydroxylase is not due to relief of inhibition of this enzyme by the reduction in concentration of the portal bile salts (4,5,6). As there is a delay of at least 24 hours in the rise in cholesterol 7 $\alpha$  hydroxylase activity upon interruption of the enterohepatic circulation (7) and since the rise is prevented by actinomycin D the increase in enzyme activity is thought to be due to induction of some component of the enzyme system or of some factor required for providing the enzyme with a supply of cholesterol rather than activation of the pre-existing enzyme.

The cholesterol 7 $\alpha$ -hydroxylase has certain features in common with several hepatic mixed function oxidases in that it requires NADPH and molecular oxygen and cytochrome P450 for its activation and it is located in the microsomal fraction. The NADPH provides a source of electrons and the flavoprotein, NADPH cytochrome P450 reductase catalyses the transfer of electrons from the NADPH to cytochrome P450. The cholesterol 7 $\alpha$  hydroxylation system resembles other hepatic microsomal mixed function oxidases in not requiring an ironsulphur protein which is essential for cytochrome P450 dependent hydroxylations occurring in the adrenal mitochondria. The iron sulphur protein is situated between the flavoprotein and cytochrome P450 (Fig. 1.3).

Although the cholesterol 7 $\alpha$ -hydroxylase has been shown to be P450 linked, no direct evidence has been presented to show that cholesterol interacts with microsomal P450 during the hydroxylation process. However the adrenal cytochrome P450 that catalyses the cleavage of the cholesterol side chain (P450<sub>scc</sub>) has been obtained

in a state of purity sufficient to show that cholesterol is interacting directly with the cytochrome (8).

The cholesterol 7 $\alpha$ -hydroxylase mixed function oxidase system also has certain differences from the rest of the hepatic mixed function oxidases. Drug treatment of the animal increases the hepatic cytochrome P450 levels with a consequent increase in the activity of mixed function oxidases that are associated with drug metabolism but this treatment has no effect on the activity of the cholesterol 7 $\alpha$  hydroxylase (9). This finding illustrates the problem that although the cholesterol 7 $\alpha$ -hydroxylase enzyme is dependent on cytochrome P450 for its activity there does not seem to be a direct relationship between hepatic cytochrome P450 concentration and cholesterol 7 $\alpha$ -hydroxylase activity. Perhaps this is explained by stating that there is a specific cytochrome P450 in liver associated with the cholesterol 7 $\alpha$ -hydroxylase enzyme system and that small alteration in its concentration cannot be detected in the presence of the general hepatic cytochrome P450. Further indications of the specificity of the cholesterol 7 $\alpha$ -hydroxylase was provided by adding radioactive cholesterol analogues to the microsomal suspension. Alteration of the  $\Delta^5$  double bond did not alter the ability of the microsomal system to 7 $\alpha$ -hydroxylate the sterol (4) but alteration of the cholesterol side chain by removing the terminal methyl group reduced the rate of 7 $\alpha$ -hydroxylation to about half that for cholesterol (10). This last result implied that the side chain of cholesterol played an important part in determining the metabolism of the whole molecule. This aspect of the enzyme action was chosen for further study and chapter 3 describes the synthesis of cholesterol analogues with longer side chains than cholesterol which were used to further define the role of the cholesterol

side chain in the metabolism of cholesterol by the liver and the adrenal mitochondria.

Traditionally, cholesterol 7 $\alpha$ -hydroxylase activity has been measured by a method which involved the addition of radioactive substrate to the microsomes and isolation of the metabolites. As the microsomes are relatively rich in cholesterol an assay based on the conversion of a small amount of exogenous cholesterol can give an indication of the activity of the enzyme but is not entirely suitable due to problems of substrate supply. This is due to the fact that the cholesterol in the microsomes exists in different pools and it is not known how interchangeable the pool contents are, or if a pool exists whose sole function is to supply substrate for the enzyme. The added tracer cholesterol could enter a pool of cholesterol that does not provide substrate and thus the calculations of a percentage conversion to 7 $\alpha$ -hydroxy cholesterol could only give an approximation of the percentage of the radioactivity accessible to the enzyme and not of the actual enzyme activity. A much improved method of measuring the activity of the enzyme would be to estimate the actual mass of product formed by the endogenous cholesterol in the microsome. This involved the use of gas liquid chromatography and mass spectrometry and has been successfully used in other laboratories (11,12). This is the obvious assay to use for study of the effect of alteration of microsomal cholesterol and an example of a similar assay is described in chapter 6.

The previously mentioned difficulty in explaining the regulation of cholesterol 7 $\alpha$ -hydroxylase by bile salts in terms of an effect on any of the known components of the enzyme system indicated that the production of 7 $\alpha$ -hydroxycholesterol from cholesterol may be

increased by increasing the supply of substrate to the enzyme. Indirect evidence of this came from the report of a 100,000 g supernatant protein which was capable of stimulating the activity of the cholesterol 7 $\alpha$ -hydroxylase (13).

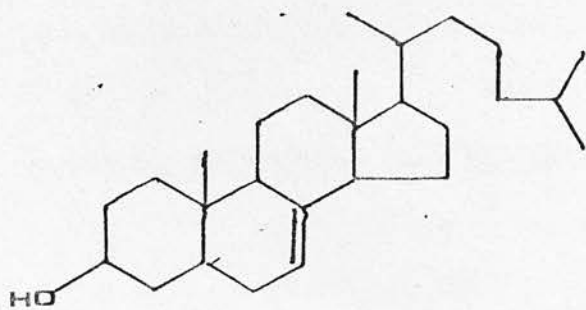
No possible mode of action of this protein is included in the report although it may act to transport cholesterol to the enzyme. A transport role for this protein implies that an interaction between the cholesterol and protein is occurring. Although there is evidence for a specific cholesterol binding protein, it is not unequivocal. Klappauf and Schubert (14) reported results of experiments with monolayers prepared from phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine and cholesterol respectively that show that Band 3 protein isolated from the erythrocyte specifically interacted with cholesterol.

Binding proteins have also been isolated from the cytosol of L cell cultures that are capable of distinguishing between cholesterol and 25 hydroxycholesterol (15). The findings raise the possibility that the activity of the cholesterol 7 $\alpha$ -hydroxylase might be regulated by a protein capable of supplying substrate to the enzyme. If cholesterol could be relocated in the microsomes to a site adjacent to the enzyme active site by such a protein, then perhaps the effect of cholestyramine feeding could be estimated in terms of the increased synthesis of such a cytosolic protein. A similar cytosolic protein has been described by Bell (16) which enhances transfer of cholesterol from the mitochondria to the microsomes. This implies that the cholesterol pools of the various cellular organelles may be capable of mutual interconversion.

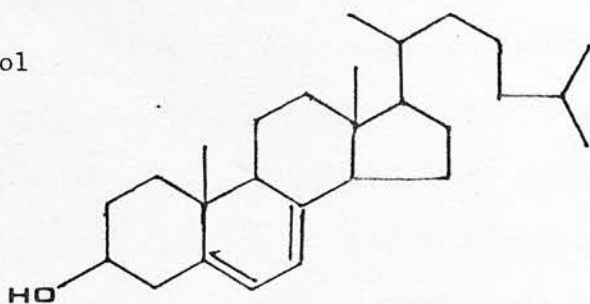
The actual function of cholesterol in a membrane has been the subject of much study in the past few years. Cellular membranes such as liver plasma membranes (17) and erythrocytes (18) contain high levels of cholesterol with the molar ratios of cholesterol to phospholipid of 0.83, 0.90 respectively while relatively low cholesterol concentrations are found in plasma membranes and subcellular membranes of nuclei, mitochondria, and microsomes from liver. The cholesterol:phospholipid ratio ranges from 0.11 to 0.33 (18) in these intracellular organelles and only free cholesterol is present in a membrane. The functions of the sterols in many biological membranes and the possible interactions with other membrane components including phospholipid and protein cannot really be studied in the intact cell due to the complexity of the system. For this reason the initial studies of the interaction of cholesterol with membrane components especially phospholipid have been performed on artificial membranes and natural membranes whose sterol content has been manipulated in some way. The model systems developed have included monolayers and liposomes and the role of cholesterol in these model membranes has been studied by several physical techniques including electron paramagnetic resonance (epr) (14), nuclear magnetic resonance spectroscopy (nmr) (20), X-ray diffraction (21), and differential scanning calorimetry (22).

When membrane lipids are spread on a film of water the amphipathic nature of membrane lipids gives rise to forces at the lipid-water interface due to hydrophilic and hydrophobic interactions. The phospholipid head group is oriented in the water phase with the hydrophobic fatty acyl chains in the air. In sterols the hydroxyl group represents the polar moiety and the sterol skeleton, with its

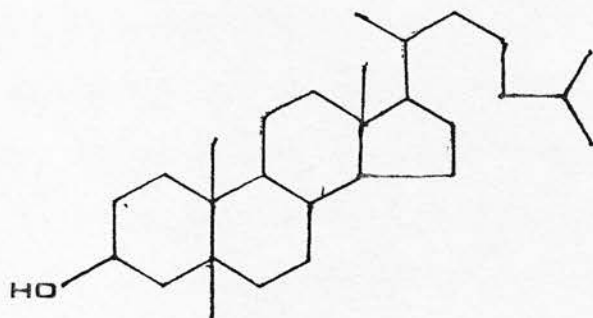




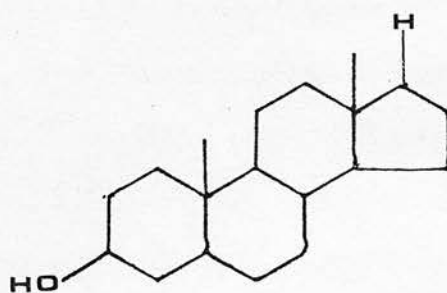
Lathosterol



7-dehydrocholesterol



coprostanol

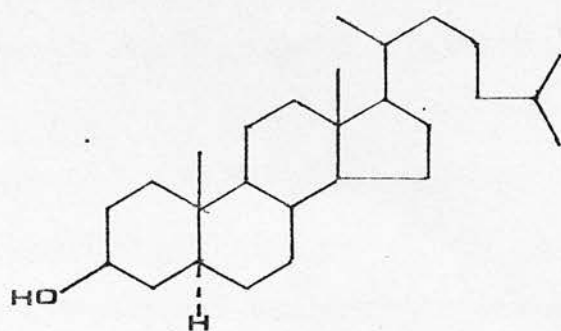


androstanol

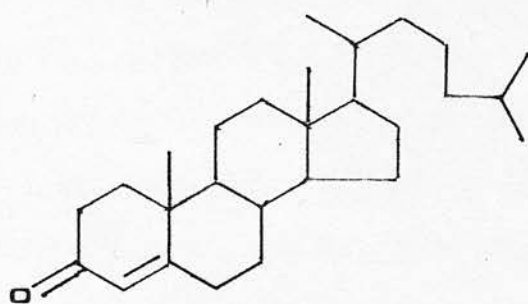
Fig. 1.1

attached side chain, the hydrophobic part. The interaction between cholesterol and phospholipid can be monitored by measuring the reduction in area of a mixed film of cholesterol and phospholipid when the film is compressed by means of a moveable barrier (23, 24). The results of these experiments showed that a planar sterol structure and a side chain of undefined length were required for the interaction of a sterol with a phospholipid and supported the view that hydrophobic interactions played an important role in cholesterol phospholipid interaction.

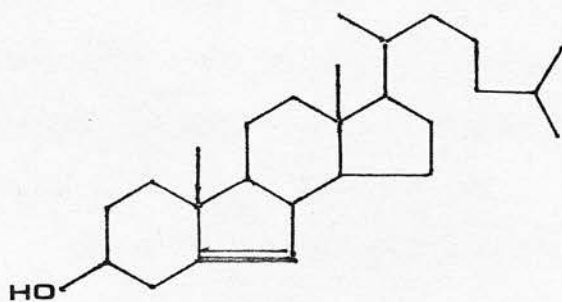
Closed lamellar structures (liposomes) consisting of layers of lipid separated by aqueous layers containing an electrolyte can be spontaneously formed after suspending dry lipid material in an aqueous solution (25). When the electrolyte containing liposomes are challenged with a hypo- or hypertonic solution the liposomes swell or shrink. Stopped flow light scattering measurements have been made on these liposomes and the optical changes observed represented the permeability of water through the outer layers of the liposome (26). A similar set of experiments have been performed by preparing liposomes containing different proportions of sterol in the presence of glucose and noting the glucose leakage by an enzymatic assay. In both sets of experiments cholesterol caused a strong reduction in the permeability of those liposomes for which an interaction of phospholipid with cholesterol was demonstrated (27, 28, 29). Lathosterol and 7-dehydrocholesterol showed similar effects to that of cholesterol, whereas stigmasterol and ergosterol were somewhat less effective. The decrease in permeability was generally proportional to the cholesterol concentration and was explained to be due to the increased packing and decreased mobility of the hydrocarbon chains. The polar head group plays a role in the sterol



cholestanol



cholest-4-en-3-one



B norcholesterol

Fig. 1.1



phospholipid interaction as the 3 $\alpha$  hydroxysterol, epicholesterol, was not able to reduce the liposome permeability (27). In agreement with the monolayer results no effect is found for compounds lacking the side chain (androstan-3 $\beta$ -ol) or with a non-planar sterol nucleus (coprostanol).

The importance of the side chain in cholesterol phospholipid interaction has been confirmed by Suckling and Boyd (30) using epr spectroscopy. Their results suggested that the steroid side chain exerted a very strong effect on its immediate environment, but were only based on experiments performed with cholesterol analogues with shorter side chains than cholesterol. It should be possible to further define the role of the cholesterol side chain in cholesterol phospholipid interaction and to determine if the iso-octane side chain confers special properties on the cholesterol molecule. Epr spectroscopy is a technique which requires the use of an external reporter group in order to adapt it to the study of membrane structure. The most common spin labels contain nitroxyl radicals which indicate the molecular motion of liposomal phospholipids. By the use of spin labelled phospholipids in which the spin label was attached at different points in the hydrocarbon chain, Hubbell and McConnell were able to show that a fluidity gradient existed in a phospholipid bilayer (31). Near the polar edge of the bilayer the motion of the phospholipid hydrocarbon chain was restricted, making this region of the membrane comparatively rigid whereas the membrane interior was in a highly fluid state with an apparent microviscosity comparable to that of a light oil. The fluidity gradient arises because the overall motion of the chain is composed of rotations about the individual C-C bonds. These rotations are transitions between trans and gauche conformations

with the gauche being of higher energy than the trans. If it is assumed that the phospholipid chains are anchored at the polar interface the chances of gauche configuration in the C-C bond increases with the length of hydrocarbon chain with a consequent increase in membrane fluidity at the bilayer centre.

The effect of cholesterol on the motion of the phospholipid chains can also be observed using epr spectroscopy and differential scanning calorimetry. Lipid bilayers composed of a single phospholipid type undergo a well defined phase transition at a characteristic temperature (phase transition temperature) from a low temperature state in which the lipid chains are in a rigid pseudo crystalline state to a high temperature form in which the chains are in a fluid state (32). Introduction of cholesterol into fluid lipid bilayers decreases the probability of gauche conformations in the lipid chains until at equimolar amounts the lipid chains are almost completely rigid in the all trans conformation (33). For non-fluid bilayers e.g. dipalmitoyl phosphatidylcholine below its phase transition, the hydrocarbon chains are in the all trans configuration and the introduction of cholesterol actually introduces gauche conformations into the chains fluidising the membrane. Thus below the transition temperature in the presence of cholesterol the lipid chains are more mobile than in the absence of cholesterol and above the transition temperature they are less mobile. The overall effect of the incorporation of cholesterol in phospholipid bilayers is to produce a state of intermediate fluidity (32). In differential scanning calorimetry, (22) the parameter that is measured is the heat absorbed by the lipid chains to induce the phase change. Although a transition from a gel to liquid crystalline state is not detectable in the presence of equimolar amounts of cholesterol by

examination of the DSC endotherm, laser Raman evidence indicates that a transition still takes place though over a very wide temperature range and it is now a non co-operative phenomenon (34).

The precise phase transition temperature for each phospholipid is a function of the unsaturation and fatty acid chain length of the phospholipid (35). When cholesterol is added to a mixture of phospholipids with different transition temperatures the cholesterol partitions with the fluid phospholipid irrespective of the nature of the other components (36). However using DSC Demel et al. (37) have shown that the cholesterol affinity for phospholipid is in the order sphingomyelin >, phosphatidylcholine > phosphatidylethanolamine. This is consistent with the observations that biological membranes containing phosphatidylethanolamine (mitochondria) contain little or no cholesterol. This is considered to be due to the fact that cholesterol and phosphatidylethanolamine cannot coexist in a bilayer structure (38). The high levels of cholesterol and sphingomyelin formed in the erythrocyte reflect the ability of cholesterol to interact with sphingomyelin. In addition the phospholipids with the highest affinity for cholesterol are located on the outer side of the membrane whereas most of the phosphatidylethanolamine and all of the phosphatidylserine is located on the inner side (39). A combination of the different affinities of the phospholipids for cholesterol and their distribution might produce cholesterol rich areas in membranes.

Recently a related study has appeared which used cholesterol oxidase (EC 11 26) to probe the organisation of a membrane. Cholesterol oxidase was found not to be capable of oxidising cholesterol present in erythrocytes and resealed erythrocyte ghosts but could gain access to the cholesterol when inside out vesicles

were prepared (40). Predominance of choline phospholipids with bulky headgroups in the outer layer of erythrocytes was suggested to provide steric hindrance to the cholesterol oxidase, whereas inner layer phospholipids did not protect the hydroxyl group of cholesterol.

The location of cholesterol in an egg yolk phosphatidylcholine liposome is suggested to be concentration dependent (41). Below 31 mole per cent the cholesterol molecules partition homogeneously into large liposomes, whereas above 31 mole per cent the cholesterol molecules with their smaller molecular dimensions pack preferentially into the areas of the bilayer with high radius of curvature (centre of bilayer).

The various physical techniques already mentioned have been used to provide information on the molecular architecture of the cholesterol phospholipid complex. Darke et al. (42) used n.m.r. to study the cholesterol phospholipid interaction and proposed that the cholesterol and phospholipid formed an equimolar complex in which apolar interactions between the steroid molecule and the first ten methylene groups of each fatty acyl chain severely restrict their motion. Under these conditions the motion of the terminal methyl groups of the fatty acyl chain was essentially unrestricted. Their model also implied that the cholesterol hydroxyl group may be hydrogen bonded to the phosphatidylcholine phosphate grouping. This binding is now considered improbable since the ester groupings of the phospholipids must be hydrated and cholesterol incorporation does not affect the  $^{31}\text{P}$  n.m.r. spectrum of various phosphatidylcholine groupings (43). The observed ability of the cholesterol to affect the motion of a section of the hydrocarbon chain has been incorporated into a model of the interaction of cholesterol and phospholipid which

does not propose the existence of a specific 1:1 complex (44).

This model proposes that the interactions between cholesterol and phospholipid fatty acyl chains in a lipid bilayer are primarily steric in nature. The carbonyl end of the fatty acyl chain parallels the cholesterol nucleus whereas the lower region of the chain terminal methyl end parallels the narrower cholesterol tail. The cholesterol molecule can limit the number of possible chain conformations along the upper part of the fatty acyl chain while simultaneously increasing the number of conformation<sup>S</sup> of the lower part of the chain. Thus the intermediate fluidity previously described (32) results due to a gradation in the steric interaction of the fatty acyl chains with the cholesterol molecule.

The motion of the isopropyl tail of the cholesterol molecule has been studied by various applications of n.m.r. spectroscopy including  $^{13}\text{C}$  n.m.r. and  $^1\text{H}$  n.m.r. While the results of both investigations indicate that the isopropyl tail undergoes rapid motion there is no agreement regarding the ability of the fatty acyl chain to restrict the cholesterol isopropyl tail motion. Opella et al. (45) regard the fact that 4G of proton irradiation was required to remove a  $^1\text{H}$ - $^{13}\text{C}$  dipolar interaction as an indication that the motion of the isopropyl tail group cannot be essentially unrestricted as indicated by the  $^1\text{H}$  n.m.r. studies of Kroon et al. (46). However both studies suggest that the 1:1 complex of phospholipid and cholesterol proposed by Darke et al. (42) is unlikely to exist.

The model of Rothman and Engelman (44) explained the observation regarding the effect of cholesterol on the molecular motion of the phospholipid fatty acyl chains but did not include a consideration of the effect of cholesterol on the phospholipid head groups. The phosphorylcholine portion of the phospholipid head group is thought



to orient parallel to the surface of the bilayer so that the positively charged N-methyl group is located close to the negatively charged phosphate on a neighbouring phospholipid in an intermolecular interaction (47). Addition of cholesterol is shown to disrupt the intermolecular interaction without altering the conformation of the polar head group that is found in pure phospholipid bilayers (48).

The spatial relationship between the cholesterol molecules has been most extensively studied by n.m.r. and X-ray diffraction (49). The best fit between the model and the measured electron density profile was obtained when the phosphorus atom was at  $23 \pm 1 \text{ \AA}$  from the bilayer centre and the cholesterol molecule was located such that its hydroxyl group was  $19.5 \pm 1.5 \text{ \AA}$  from the bilayer centre. Assuming that the phospholipid head group is in the bilayer plane this result placed the hydroxyl group in the region of the bilayer adjacent to the ester carbonyl oxygen. The involvement of hydrogen bonds between the cholesterol hydroxyl group and the carbonyl group has been implicated in cholesterol: phospholipid interactions, but was recently conclusively proved by Schwarz and Paltauf (50) by comparison of the passive diffusion of  $\text{Na}^+$ ,  $\text{Cl}^-$  and glucose across the membrane of single walled liposomes formed from cholesterol and diether phosphatidylcholine (which contains no ester carbonyl group), 1-ether-2-ester phosphatidylcholine and diester phosphatidylcholine. The replacement of the ester groups by ether groups which cannot hydrogen bond removed the cholesterol mediated reduction in the glucose permeability previously observed by Demel et al. (27). The hydrogen bonding can only take place when the sterol contains an equatorial hydroxyl group ( $3\beta$ ). An axial hydroxyl group ( $3\alpha$ ) cannot hydrogen bond with

carbonyl oxygen because the O-H-O bond angle is not consistent with hydrogen bond formation.

The most recent model of the interaction between cholesterol and phospholipid has been proposed by Huang (51). As in the model proposed by Rothman and Engelman the interaction is seen as being primarily hydrophobic in nature. In addition the nature of the phospholipid involved in the interaction is defined as consisting of two fatty acyl chains with the saturated acid at position (1) and the unsaturated acid at position (2).  $C_3$  of cholesterol is placed about the same level of  $C_2$  of the adjacent fatty acid in order to allow the possibility of hydrogen bonds between the cholesterol and the phospholipid molecules. In the model of Brockerhoff (52), which implies that hydrogen bonds are the most important feature in defining the interaction between cholesterol and phospholipid, membranes are visualised as consisting of a hydrophobic core, two hydrogen <sup>bond</sup> belts and two polar zones. This model suggests that cholesterol forms a hydrogen bond with the carbonyl group at position 2 whereas the Huang model indicates a hydrogen bond in this position is unlikely because the unsaturation ensures that this chain is packed preferentially to the  $\beta$  surface of the cholesterol nucleus rendering the hydroxyl on the  $\alpha$  face unavailable for bonding.

The saturated fatty acyl chain of the phosphatidylcholine is proposed to pack in close contact with the  $\alpha$  face of the steroid nucleus. The unsaturated  $C_{18:1}$  chain at position 2 of phospholipid in the bilayer appears to consist of two linear parallel straight segments linked by a  $\Delta$  trans-gauche kink, the length of each segment depending on the position of the gauche conformation. A 9, 10 trans-gauche link represents a gauche conformation between  $C_{11}$  and  $C_{12}$  of

Fig. 1.4

	Å from bilayer centre
C <sub>27</sub> , C <sub>26</sub>	1.5
C <sub>25</sub>	3
C <sub>24</sub>	4
C <sub>21</sub>	8
C <sub>19</sub>	9

A schematic diagram to show the relative location of cholesterol and phosphatidylcholine in a bilayer.



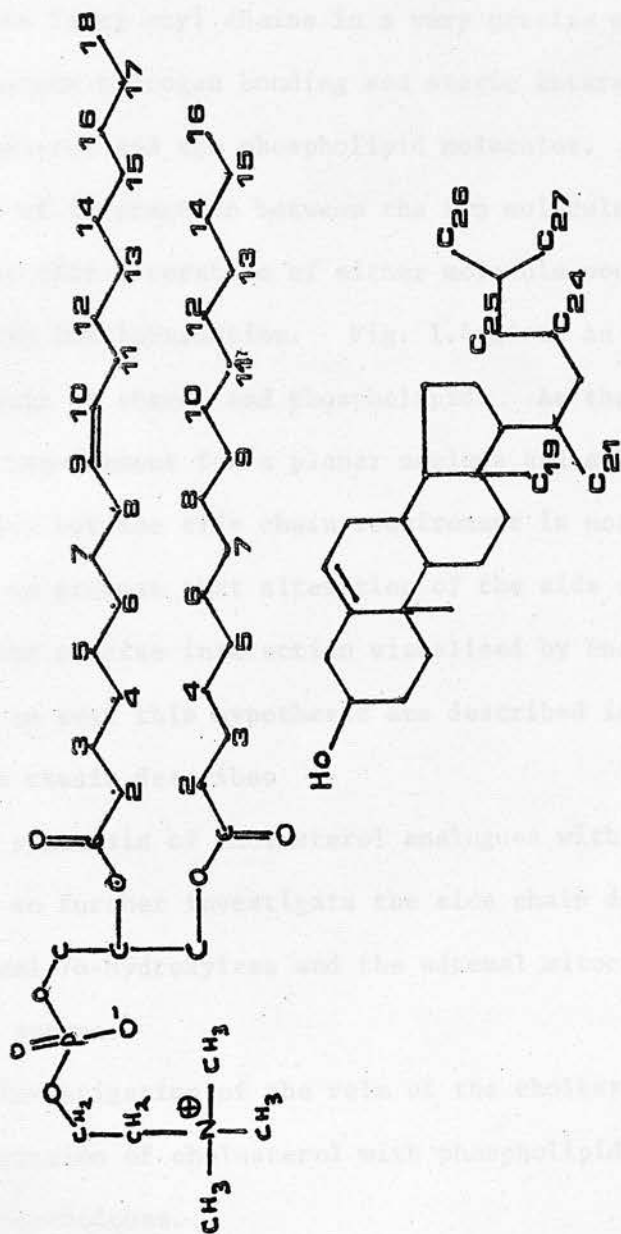


Fig. 1.4

the fatty acid generating a  $10\text{\AA}$  hydrophobic pocket which is sufficient to fit the packing requirements of the 2 angular methyl groups at  $C_{18}$  and  $C_{19}$  on the  $\beta$  face of the cholesterol nucleus.

Thus the model visualises that the cholesterol is fitting in between the fatty acyl chains in a very precise manner which allows maximum hydrogen bonding and steric interaction between the cholesterol and the phospholipid molecules. This apparent precision of interaction between the two molecules would tend to suggest that alteration of either molecule would result in a decrease in the interaction. Fig. 1.4 gives an indication of spatial relationship of sterol and phospholipid. As the sterol has an absolute requirement for a planar nucleus and a  $3\beta\text{OH}$  group for interaction but the side chain requirement is not so rigorously defined, we propose that alteration of the side chain should disrupt the precise interaction visualised by Huang. Experiments designed to test this hypothesis are described in chapters 4 and 5.

This thesis describes

- (1) The synthesis of cholesterol analogues with altered side chains in order to further investigate the side chain dependence of cholesterol  $7\alpha$ -hydroxylase and the adrenal mitochondrial side chain cleavage enzyme.
- (2) An investigation of the role of the cholesterol side chain in the interaction of cholesterol with phospholipid by a selection of physical techniques.
- (3) An attempt to gain more information regarding the supply of substrates for the cholesterol  $7\alpha$ -hydroxylase.
- (4) An investigation of the differences that exist between the cholesterol  $7\alpha$ -hydroxylase and the drug metabolising enzymes of the liver.

2.1 Animals and diets

Male and female rats of the Wistar strain were used and weighed approximately 350 g. They were obtained from the Bush Medical Animal Breeding Centre of Edinburgh University. Control diets consisted of 10% wholemeal flour, 35% skimmed milk powder and 55% dried yeast. Except where statements to the contrary are made, this basal diet was supplemented with the bile acid sodium taurochenodeoxycholate at a level of 42 w/w.

CHAPTER 2

Phenobarbitone (PB) 1-methyl-3-isobutyl-5-methylxanthine (3-MX) and pregnenolone 16 $\alpha$ -carboxylate (PCN) were administered to increase the amount of cytochrome P450 present in the microsomes. The phenobarbitone was added to the drinking water at a concentration of 1 mg/ml for 10 days prior to sacrifice whereas the PCN and 3-MX were injected intraperitoneally.

MATERIALS and METHODS

2.2 Preparation of the livers

In order to reduce the amount of haemoglobin present in the microsomal fractions, the rats were subjected to ether anaesthesia, and a needle connected to a reservoir of cold isotonic potassium chloride was inserted into the hepatic portal vein. The perfusion was completed in 3 minutes.

2.3 Preparation of microsomes

The protocol followed was essentially that described by Ernster et al. (39). The perfused liver was excised and sliced with scissors and homogenized by four passes of a 'Teflon' pestle in 0.25M potassium chloride (pH 7.4) at 4°C. The homogenate was centrifuged for 20 minutes at 17,500 x g to remove large particles. The supernatant from this centrifugation was re-centrifuged at 100,000 x g for 1 hour. The pellet formed contained the endoplasmic reticulum and a large amount of glycogen,

## 2.1 Animals and their diets

Male and female rats of the Wistar strain were used and weighed approximately 150 g. They were obtained from the Bush Small Animal Breeding Centre of Edinburgh University. Control diets consisted of 70% wholemeal flour, 25% skimmed milk powder and 5% dried yeast. Except where statements to the contrary are made, this basic diet was supplemented with the bile acid sequestering resin cholestyramine at a level of 4% w/w. Phenobarbitone (Pb) 3-methyl cholanthrene (3 MC) and pregnenolone 16 $\alpha$ -carbonitrile (PCN) were administered to increase the amount of cytochrome P450 present in the microsomes. The phenobarbitone was added to the drinking water at a concentration of 1 mg/ml for five days prior to sacrifice whereas the PCN and 3 MC were injected intraperitoneally.

## 2.2 Perfusion of the livers

In order to reduce the amount of haemoglobin present in the microsomal fraction, the rats were subjected to ether anaesthesia, and a needle connected to a reservoir of cold isotonic potassium chloride was inserted into the hepatic portal vein. The perfusion was completed in 2 minutes.

## 2.3 Preparation of microsomes

The protocol followed was essentially that described by Ernster et al. (53). The perfused liver was excised and diced with scissors and homogenised by four passes of a 'Teflon' pestle in 0.154 M potassium chloride (4 vols/g wet weight tissue). The homogenate was centrifuged for 20 minutes at 17,500 x g to remove large particles. The supernatant from this centrifugation was recentrifuged at 100,000 x g for 1 hour. The pellet formed contained the endoplasmic reticulum and a large amount of glycogen,

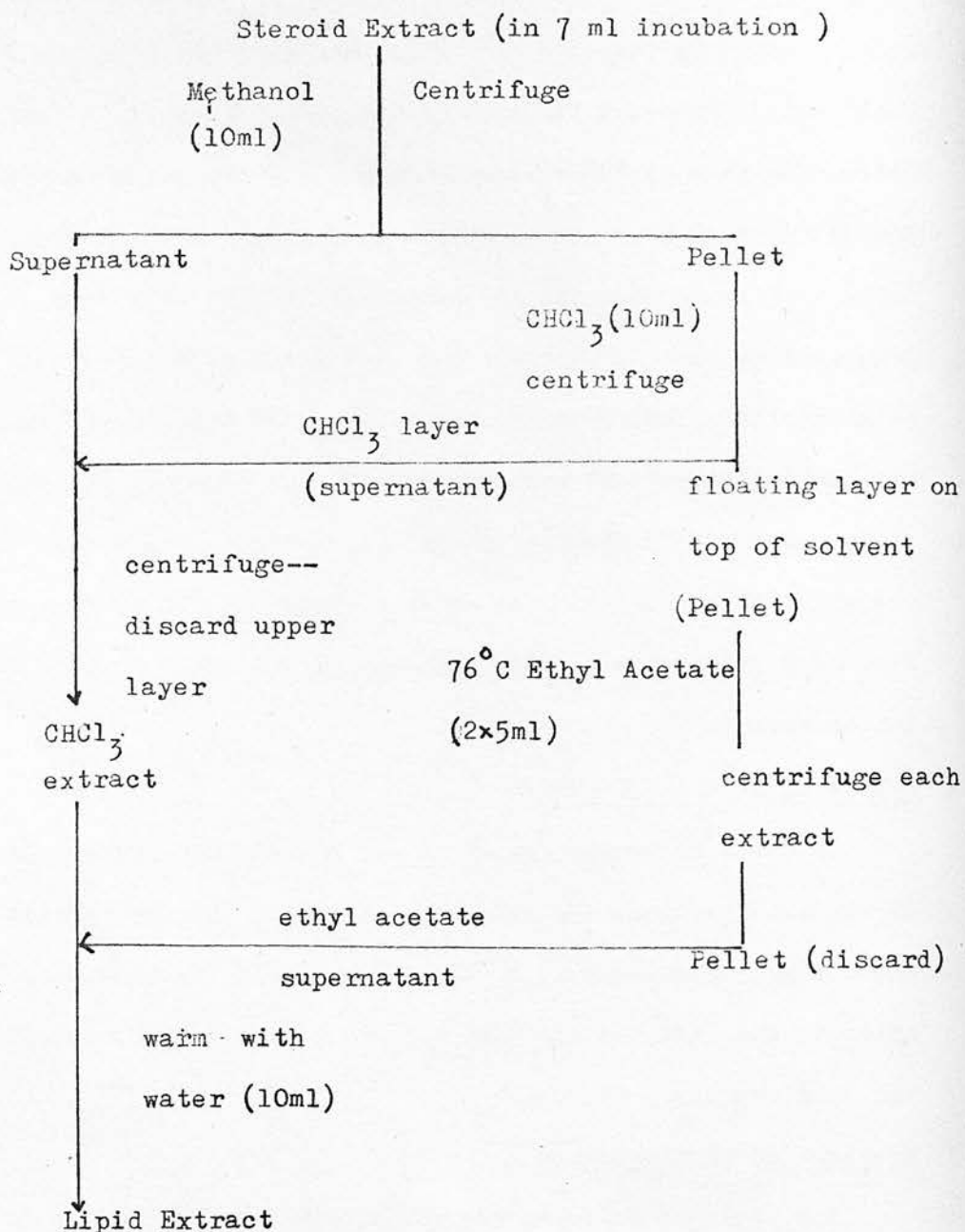


Fig. 2.1

Fig. 2.1

Lipid extraction procedure used in the cholesterol 7 $\alpha$  hydroxylase assay.

and was resuspended in distilled water, freeze dried, and stored at  $-20^{\circ}\text{C}$ .

#### 2.4 Preparation of a butanol powder of rat liver microsomes

About 2 g of the lyophilised powder were homogenised in 400 ml of n-butanol cooled to  $-20^{\circ}\text{C}$ . The homogenate was centrifuged at  $17,500 \times g$  rpm in an MSE 1800 at  $-20^{\circ}\text{C}$ , in a rotor, which had been precooled overnight at  $-20^{\circ}\text{C}$ , for 5 minutes. The supernatant was discarded and the pellet was resuspended in 400 ml acetone at  $-20^{\circ}\text{C}$  and recentrifuged at  $17,500 \times g$  for 5 minutes. The resulting pellet was once more resuspended in 100 ml of acetone at  $-20^{\circ}\text{C}$  and filtered on a Buchner Funnel. The powder was washed with 50 ml cold ( $-20^{\circ}\text{C}$ ) acetone and dried under vacuo at room temperature for 1 hour. A powder thus prepared could be stored at  $-20^{\circ}\text{C}$  for two months with no apparent loss of cholesterol  $7\alpha$ -hydroxylase activity, whereas freezing the microsomal pellet itself results in a complete loss of activity on thawing. Vore et al. (54) found that this procedure removes all of the neutral lipid including cholesterol, 80% of the phosphatidylcholine and phosphatidylethanolamine, and 75% of the phospholipid phosphorus. In addition the anhydrous conditions employed ensured a minimal loss of cytochrome P450 and NADPH cytochrome P450 reductase.

#### 2.5 Assay of cholesterol $7\alpha$ -hydroxylase

##### (a) The cholesterol $7\alpha$ -hydroxylase assay

$[4-^{14}\text{C}]$  cholesterol (specific activity 50 mCi per mmole) was purified by T.L.C. using a solvent system of petroleum ether:di-isopropyl ether:acetic acid 30:70:2, prior to use. About 1 mg (0.1  $\mu\text{Ci}$ ) radioactive cholesterol, dissolved in 50  $\mu\text{l}$  acetone was added to the incubation medium of 7 ml of 0.1 M potassium phosphate buffer pH 7.4 containing 10 mM  $\beta$ -mercaptoethylamine (cysteamine),



5  $\mu$ moles NADP, 50  $\mu$ moles glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase and approximately 10 mg microsomal protein, which is approximately equivalent to 1 g wet weight of liver. These are the optimal conditions for the assay as described by Boyd et al. (9). Cysteamine acts to prevent autoxidation of substrate cholesterol. After incubation for 1 hour at 37°C with constant agitation, the reaction was terminated by the addition of 5 ml of methanol. The methanolic incubation solution was then transferred to lipid extraction tubes and the incubation tubes were rinsed with a further 5 ml of methanol and the two solutions were combined together. The radioactive sterols were then extracted by a neutral lipid extraction scheme presented in Fig. 2.1. The organic extracts were evaporated under a stream of air and the residues applied to Keisegel H Type 60 thin layer chromatography plates. The reaction products were separated from the substrate by developing in a solvent system of benzene:ethyl acetate (7:13) and the exact location of the radioactive metabolites was identified by scanning with a Panax thin layer scanner. In the absence of cysteamine, 7-keto-cholesterol, 7 $\beta$  and 7 $\alpha$  hydroxycholesterol and cholestan-3 $\beta$ , 5 $\alpha$ , 6 $\beta$  triols are formed (55), but under the conditions described, only one major metabolite is formed. The silica gel corresponding to radioactive peaks was scraped into counting vials and 5 ml of scintillants added (20 g PPO, 150 mg POPOP in 5 litres toluene).

Radioactivity was then quantitatively determined in a Packard Tri-Carb liquid scintillation counter. The presence of silica gel does not cause significant quenching, and the efficiency of counting  $^{14}\text{C}$  is approximately 80 per cent.



<u>Typical recoveries of (<math>4^{14}\text{C}</math> cholesterol ) from</u>		
<u>an incubation.</u>	c.p.m.	recovery
Standard $4^{14}\text{C}$ cholesterol	15,000	100
After a lipid extraction	14,100	94
After T.L.C.	11,400	76
After a methanol extraction	7,500	50
from Kieselgel H		

Table 2.1

When the metabolism of the tritiated cholesterol side chain analogues by the  $7\alpha$ -hydroxylase system was measured, the radioactive metabolites formed were separated by thin layer chromatography. As this procedure yields many products the chromatograms were scraped in centimetre bands.

(b) The determination of  $7\alpha$ -hydroxycholesterol

Incubations of liver microsomes or cholesterol depleted microsomes were conducted as previously described but without the addition of  $[4-^{14}\text{C}]$ -cholesterol. After stopping the reactions with methanol  $[7^3\text{H}]$ - $7\alpha$  hydroxycholesterol was added to each flask. Lipids were extracted and subjected to TLC and standard  $7\alpha$  hydroxycholesterol was run at the edge of each plate. This was located by spraying with phosphomolybdic acid. A bright turquoise colour is obtained on gentle heating. The area of the plate corresponding to  $7\alpha$ -hydroxycholesterol was removed into a centrifuge tube and the silica gel was extracted twice with methanol. An aliquot of the methanol was transferred to a scintillation vial, evaporated, and counted in a Packard liquid scintillation spectrometer. This gave an estimation of the recovery process. The remainder was taken to dryness under nitrogen and a known quantity of cholesterol was added as an internal standard for gas liquid chromatography (GLC). A sample of this mixture was injected onto the GLC column in redistilled acetone (the conditions for chromatography are described below). The  $7\alpha$ -hydroxycholesterol in the sample was quantified by comparing the ratio of the heights of the peaks obtained for the  $7\alpha$ -hydroxycholesterol and cholesterol with a standard curve of mass ratio versus peak height ratio. This value is corrected for recovery losses and hence the mass of  $7\alpha$ -hydroxycholesterol formed during the incubation was calculated.

Fig. 2.2

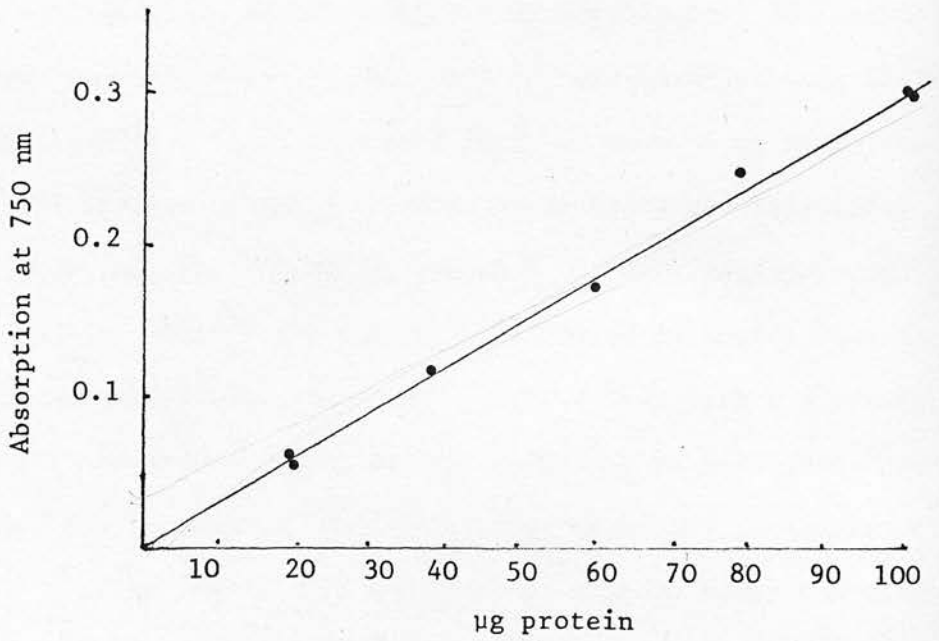
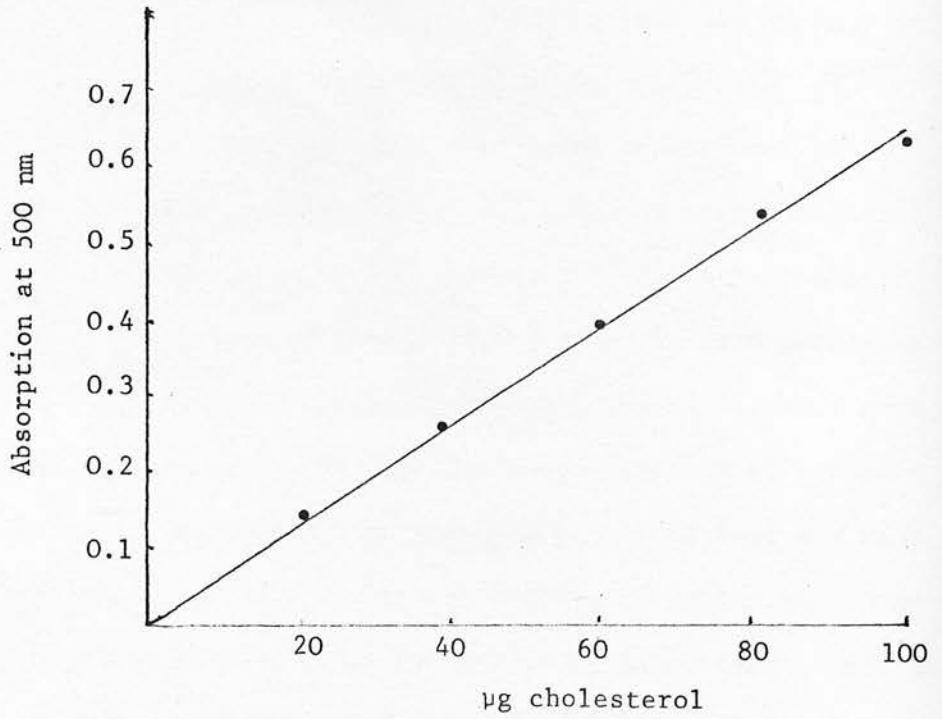


Fig. 2.4

Fig. 2.2

Cholesterol calibration curve.

Fig. 2.4

Protein calibration curve.

(c) Conditions for 7 $\alpha$ -hydroxycholesterol determinations by GLC

All gas chromatographic work was carried out on a Pye 104 gas chromatograph equipped with a flame ionisation detector. The columns used were 1.75 metres long of 4 mm internal diameter glass tubing silanised with dichloro-dimethylsilane. The liquid phase used was 1% SE 30 on 100-120 mesh gas chrom Q. The column oven temperature was 205°C, the injection port temperature was 220°C, and the detector oven temperature was 240°C. Nitrogen was used as a carrier gas at a flow rate of 30 ml per minute. Using these conditions and an attenuation setting of  $20 \times 10^2$ , a full scale deflection of the pen recorder represented the presence of 0.8  $\mu$ g of 7 $\alpha$ -hydroxycholesterol.

2.6 Cholesterol assay

The levels of cholesterol in microsomal preparation were measured in one of two ways:

- (a) by gas liquid chromatography
- (b) by the use of cholesterol oxidase prepared from *Nocardia* species

(a) This procedure involved a lipid extraction, similar to that described by Folch (56), thin layer chromatography in petroleum ether:di-iso-propyl ether:acetic acid (70:30:2v/v) and elution of the cholesterol (located by including a sample of radioactive cholesterol in the microsomal solution prior to the lipid extraction) from the gel with methanol. After removal of an aliquot of the methanol to measure the recovery of label, this final extract was evaporated to dryness and then redissolved in redistilled acetone in the presence of pregnenolone as an internal standard. Average recoveries are noted on the opposite page (Table 2.1). The sample was then applied to a column of 100-120 mesh gas chrom Q on which was adsorbed 1 per cent SE 30. With the column oven at 238°C, the

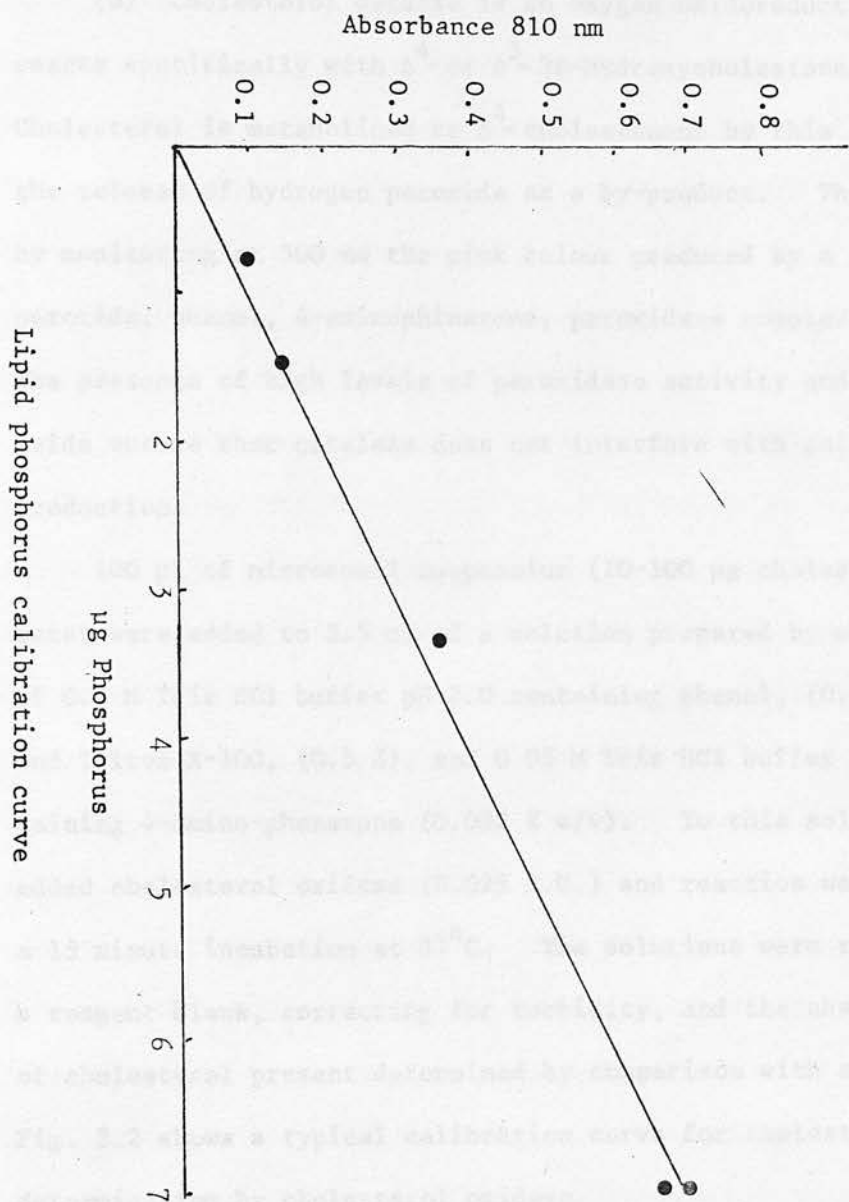


Fig. 2.3



injection heater at 300°C and a nitrogen flow of 30 cm/minute the amounts of cholesterol present in the sample were determined by comparison of the peak height ratios for the unknown cholesterol and known pregnenolone acetate with standard mixtures of the two components.

(b) Cholesterol oxidase is an oxygen oxidoreductase which reacts specifically with  $\Delta^4$ - or  $\Delta^5$ -3 $\beta$ -hydroxycholestanes. Cholesterol is metabolised to  $\Delta^4$ -cholestenone by this enzyme with the release of hydrogen peroxide as a by-product. This is measured by monitoring at 500 nm the pink colour produced by a hydrogen peroxide, phenol, 4-aminophinazone, peroxidase complex (57). The presence of high levels of peroxidase activity and sodium azide ensure that catalase does not interfere with colour production.

100  $\mu$ l of microsomal suspension (10-100  $\mu$ g cholesterol) or water were added to 2.5 ml of a solution prepared by mixing equal volumes of 0.4 M Tris HCl buffer pH 7.0 containing phenol, (0.144 % w/v), and Triton X-100, (0.5 %), and 0.05 M Tris HCl buffer pH 7.0 containing 4-amino-phenazone (0.022 % w/v). To this solution, was added cholesterol oxidase (0.025 E.U.) and reaction was promoted by a 15 minute incubation at 37°C. The solutions were read against a reagent blank, correcting for turbidity, and the absolute amounts of cholesterol present determined by comparison with standards. Fig. 2.2 shows a typical calibration curve for cholesterol determination by cholesterol oxidase.

## 2.7 Phospholipid determination

Phospholipid was measured in two ways:

- (1) by a modification of the methods of Zilversmit and Davis (58)
- (2) by the method of McClare (59)

(a) Phospholipids, proteins and nucleic acids were precipitated from the sample by the addition of a 10% (w/v) solution of trichloroacetic acid and a subsequent 30 min incubation at 4°C. Centrifugation of the precipitate yielded a pellet which was separated from the supernatant and vortexed with a chloroform: methanol solution (2:1) to separate the lipids from the rest of the material. The organic phase thus obtained was blown down to dryness under nitrogen. The extract was then digested for an hour by boiling with (1) 1 ml perchloric acid or (2) 0.5 ml perchloric acid respectively.

(b) The method of detection of the phosphate liberated by this procedure differed in both cases.

(1) Zilversmit added 3.5 ml water followed by 0.5 ml 8.3% ammonium molybdate and 1 ml of 1% amidol in 20 percent sodium metabisulphite. After exactly 10 minutes the absorbance at 720 nm against a perchloric acid boiled water blank was determined. This colour reaction was useful in the range 1 to 25 µg phosphorus.

(2) The McClare method used triphenyl phosphine instead of  $\text{Na}_2\text{HPO}_4$  and involved the use of a freshly prepared colorimetric reagent containing 8% perchloric acid, 1% ammonium molybdate and 0.2% ascorbic acid. 9.5 ml of this reagent were added to each digest tube which ~~was~~ then covered and heated for 40 minutes in a 50°C water bath. The phosphate content was measured by absorbance against a reagent blank at 825 nm and converted to µg phospholipid by multiplication by 25. This colour reaction was useful in the range 1-7 µg phosphorus. Fig. 2.3 shows an example of a standard curve for phosphate determination.

Of the two methods McClare's was preferable due to its greater sensitivity, its reproducibility and the stability of the coloured complex produced.

2.8 SDS polyacrylamide gel electrophoresis was performed according to Weber and Osborn (60).

2.9 Protein was assayed either according to the method of Lowry (61) or by the biuret method (62). Fig. 2.4 shows a typical Lowry calibration curve.

#### 2.10 Ethylmorphine-N-demethylase assay

Ethylmorphine is N-demethylated by liver microsomes in the presence of an NADPH generating system with the concomitant release of formaldehyde.

Liver microsomes were suspended at a final concentration of 2 mg microsomal protein per ml in a final total volume of 10 ml of 50 mM Tris, 150 mM KCl, and 10 mM  $\text{MgCl}_2$ , pH 7.4 containing 8 mM ethylmorphine. After equilibration at  $37^\circ\text{C}$  for two minutes, 0.2 ml of a NADPH generator (7.5 mg/ml  $\text{NADP}^+$ , 25 mg/ml glucose phosphate and 5 iu/ml glucose-6-phosphate dehydrogenase) was added to the flask to initiate the reaction. 2 ml samples were taken at zero time, 5 minutes and 10 minutes and immediately added to centrifuge tubes containing 2.0 ml 15% trichloroacetic acid. After a 10 minute centrifugation 2.0 ml of the supernatant was transferred into 2 ml of Nash reagent (2 M ammonium acetate, 50 mM acetic acid and 20 mM acetyl acetone) and colour development was promoted by an eight minute incubation at  $58^\circ\text{C}$ . The absorbance at 412 nm was measured and converted into the concentration of formaldehyde present by use of an absorbance coefficient of  $8 \text{ mM}^{-1} \text{ cm}^{-1}$ .

## 2.11 Assay of cytochrome P450

Cytochrome P450 concentration was determined essentially as described by Omura and Sato (63) in a Pye Unicam SP 800 double beam spectrophotometer. Thus to approximately 6 ml of sample (12 mg protein) were added a few crystals of sodium dithionite and the suspension was split into two fractions, a sample and reference fraction which were initially balanced at 500 nm. After recording a base line of equal absorbance, carbon monoxide was gently bubbled through the cuvette for about 30 seconds. A recording was then made of the CO reduced minus reduced difference spectrum and the concentration of cytochrome P450 calculated by measuring the absorbance at 450 nm calculated by measuring the absorbance at 450 nm relative to 490 nm using an extinction coefficient of  $91 \text{ mM}^{-1} \text{ cm}^{-1}$ .

## 2.12 Measurement of cytochrome $b_5$

The cytochrome  $b_5$  concentration was determined by observing the difference in absorbance between the peak at 424 nm and the trough at 409 nm obtained when a few grains of NADPH are added to the sample cuvette. Using this reduced minus oxidised spectrum and an extinction coefficient of  $185 \text{ mM}^{-1} \text{ cm}^{-1}$  the cytochrome  $b_5$  concentration present was calculated.

## 2.13 Assay of NADPH-cytochrome c oxidoreductase

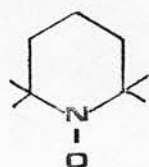
To 218 ml of 0.1 potassium phosphate buffer pH 7.7 were added 50  $\mu\text{l}$  i.e. 1 mg cytochrome c, 100  $\mu\text{l}$  of NADPH generator (0.5  $\mu\text{moles}$   $\text{NADP}^+$ , 5  $\mu\text{moles}$  glucose-6-phosphate dehydrogenase). The reaction performed at  $25^\circ\text{C}$  was started by addition of the flavoprotein containing sample. The velocity of reaction was measured in a Pye-Unicam SP 800 double beam spectrophotometer in the dual wavelength mode. The difference in absorbance between 551 nm and 540 nm was

recorded as a function of time. An extinction coefficient of  $19 \text{ mM}^{-1} \text{ cm}^{-1}$  was used as the difference between reduced and oxidised cytochrome c.

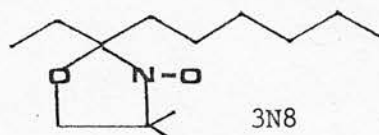
#### 2.14 Preparation of single walled liposomes

Liposomes were prepared by the evaporation of a chloroform solution of the lipids to form a thin film. The vessel containing this film was then placed in a vacuum dessicator for two hours during which time any residual chloroform was removed. The lipid film was converted into multilayered liposomes by vortexing for 5 minutes with sufficient 10 mM Tris HCl pH 7.4, 1 mM EDTA to prepare a 3 mg/ml lipid dispersion. In order to prepare single walled liposomes 5 ml of the multilayered liposomes (3 mg/ml) were subjected to a sonication step (using a Rapibus 150 sonicator, output 20 w). This involved a 40 minute treatment (16 x 2 minute bursts with 30 seconds between each burst) at power level 8 to 9 with a 9 mm diameter titanium probe. The solution to be sonicated was placed in a standard ice jacketed bottle (2 cm diameter, length 9 cm). After the sonication step the single walled liposomes were separated from titanium fragments and undispersed lipid by centrifugation of the optically clear preparation at 100,000 g for 30 minutes. The liposomes thus obtained were shown to be free of lysolecithin and could be stored for a week at  $4^{\circ}\text{C}$  without any visible precipitation occurring.

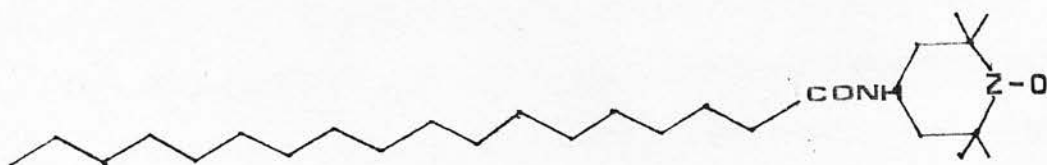
Using this sonication procedure the liposomes were shown to form a homogeneous population as judged by the elution profile from a Sepharose 4B column (64). No material was present at the void volume of this column.



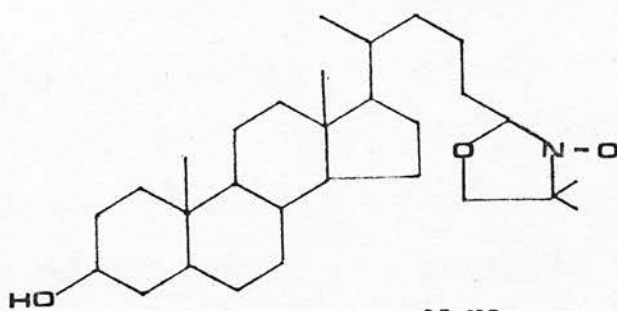
Tempo



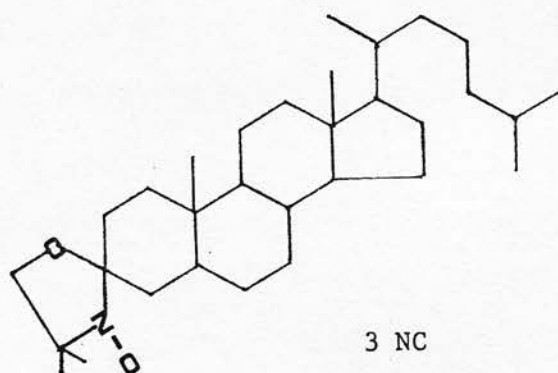
3N8



INS



25 NC



3 NC

Fig. 2.5



Fig. 2.5

Structures of the spin labels used in this study.

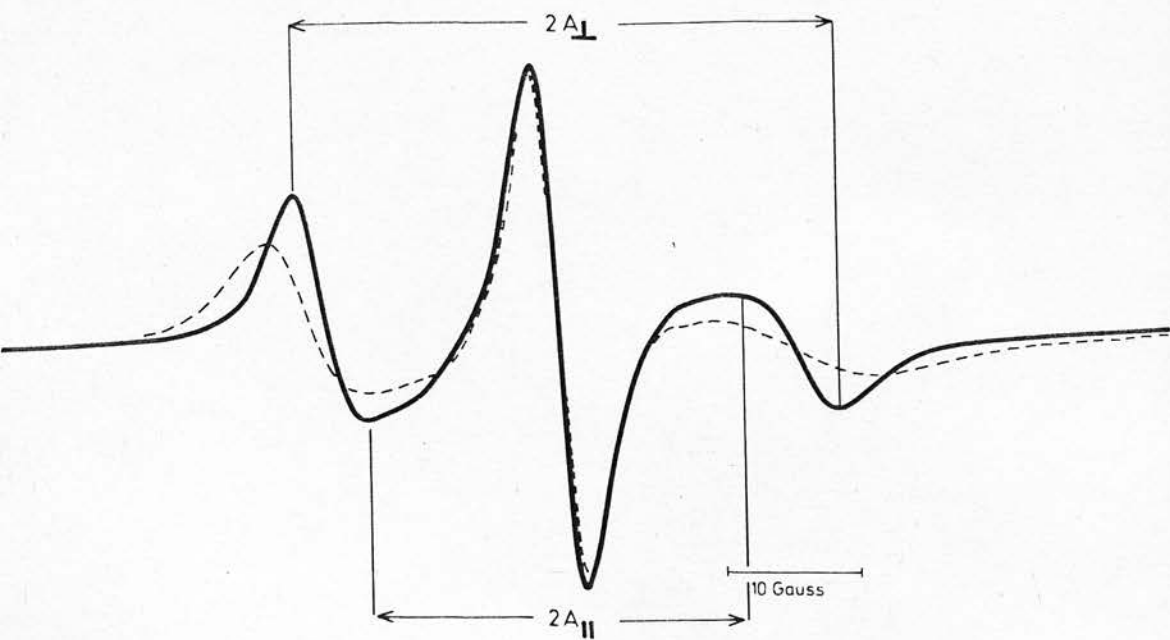
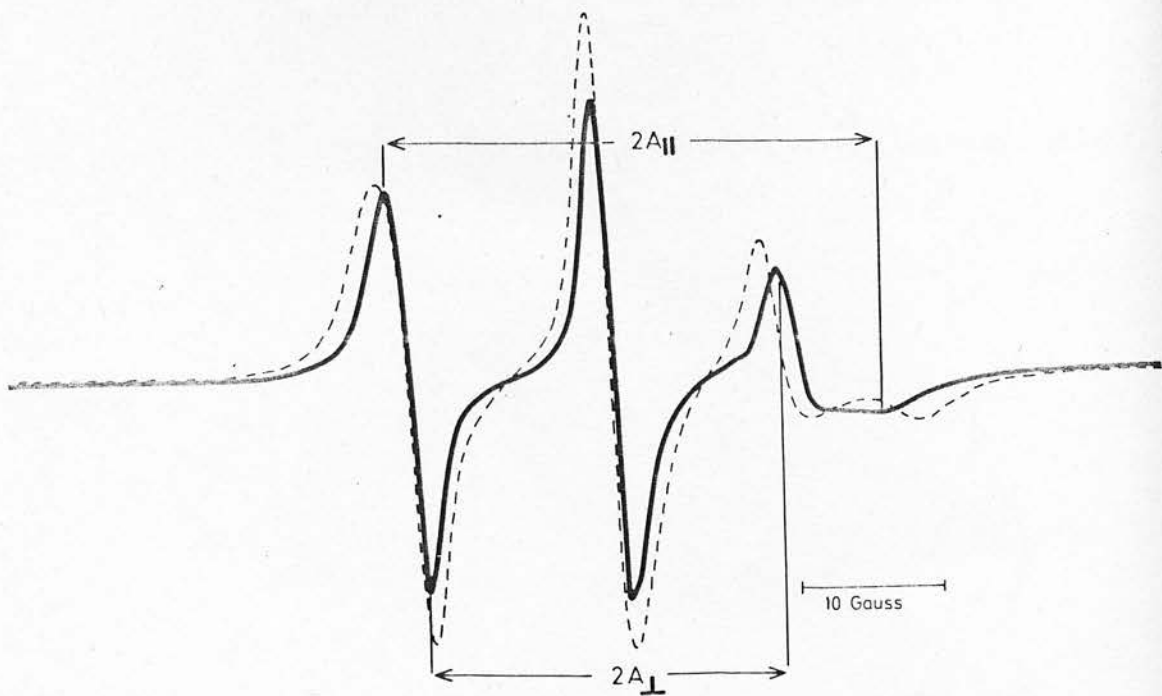


Fig. 2.6 (a)

Spin label spectra at 25 NC in the presence (40 mole % (---)) or absence (—) of cholesterol. The order parameter (S) is calculated thus

$$S = \frac{2A_{11} - 2A_1}{25}$$

Fig. 2.6 (b)

Spin label spectra of 3 NC in the presence (40 mole% (----)) or absence (—) of cholesterol. The order parameter (S) is calculated thus

$$S = \frac{2A_1 - 2A_{11}}{25}$$

## 2.15 Preparation of egg yolk phosphatidylcholine

Phosphatidylcholine was prepared from egg yolks by the method of Pangborn (65), which involved an initial acetone extraction which removed all of the neutral lipids leaving a cake of protein and phospholipids. A 95 per cent ethanol extraction of the cake separated the protein from phospholipids which were subdivided by a combination of solvent extraction methods and the formation of cadmium adducts of the phosphatides. Sphingomyelin was retained with the phosphatidylcholine during this preparation but was eventually removed by precipitation from a cold ether solution of phosphatidylcholine. Using this procedure it has been shown that the phosphatidylcholine thus obtained contained a trace amount of cadmium detectable by microelectrophoresis (66) (but not by 5% Ag NO<sub>3</sub>), however this was not thought to be of critical importance.

Thin layer chromatography shows only one spot when the chromatogram was developed by chloroform:methanol:water (65, 35, 5 v/v).

## 2.16 Electron spin resonance

### Spin labelling experimental details

#### (a) 3NC and 25NC spin labels (for structure see Figure 2.5)

Multilayered liposomes were prepared as previously described using 0.154 M KCl (20  $\mu$ l) instead of 10 mM Tris-HCl-1mM EDTA. They contained the required proportions of sterol, egg yolk phosphatidylcholine and 1 mole % of the steroid spin label. (As a high concentration of spin label masks the spectrum it was essential to use a spin label concentration of less than  $10^{-3}$  M). Electron spin resonance spectra were recorded on a Varian E4 spectrometer (at the X band (3272 G) with scan range 100 G, modulation frequency 100 kHz, modulation amplitude 1.25 G, time constant 1 second, at a power input of 20 mW, with the klystron tuned at 9.15 GHz) within two hours of preparation at 30°C with the samples in haematocrit

capillary tubes sealed at one end. Order parameters for 3NC were calculated according to Griffith et al. (67) and Smith (68). The three line spectrum obtained in both cases is the result of averaging of the orientations of the principal axes of the spin label group relative to the applied magnetic field, Fig. 2.6 shows representative spin label spectra. In one orientation along the Z axes of the nitroxide an ESR spectrum of three narrow lines separated by 32 G would be observed whereas along the x and y axes a sharp three lines spectrum separated by approximately 6 G would be recorded. The order parameter (S) is given by the equation which is uncorrected for polarity effects.

$$(S) = (A_{\max} - A_{\min}) / A_{zz} - \frac{1}{2} (A_{xx}^c + A_{yy}^c)$$

$$= A_{\max} - A_{\min} / 25 \text{ G.}$$

For (25NC)  $A_{zz}^c - \frac{1}{2} (A_{xx}^c + A_{yy}^c)$  is also estimated to be 25 G from its spectrum in chloroform at  $-172^\circ\text{C}$  (68). 25 G is the figure used to calculate (S) in Chapter 4.

(b) 2,2,6,6 Tetramethylpiperidine-I-oxyl (Tempo)

A chloroform solution of sterol and a saturated phosphatidylcholine (dipalmitoylphosphatidylcholine) was evaporated under nitrogen forming a film on the sides of a 3 ml test tube. The remaining solvent was removed by a 2 hour vacuum dessication, 0.154 M KCl (40  $\mu\text{l}$ ) was added and the solution was heated to  $60^\circ\text{C}$  for 30 seconds in order to melt the saturated phospholipid. The resulting solution was vortexed for 30 seconds and the process was repeated 4 times. Tempo (1  $\mu\text{l}$  in aqueous solution  $4 \times 10^{-8} \text{ M}$ ) was then added, and the mixture was reheated to  $60^\circ\text{C}$  for 15 seconds, vortexed for 1 minute and placed in a flat cell. The plane of the cell was placed transverse to the length of cavity so as to locate the whole of the sample in a region of minimum electric and maximum magnetic field thus minimising

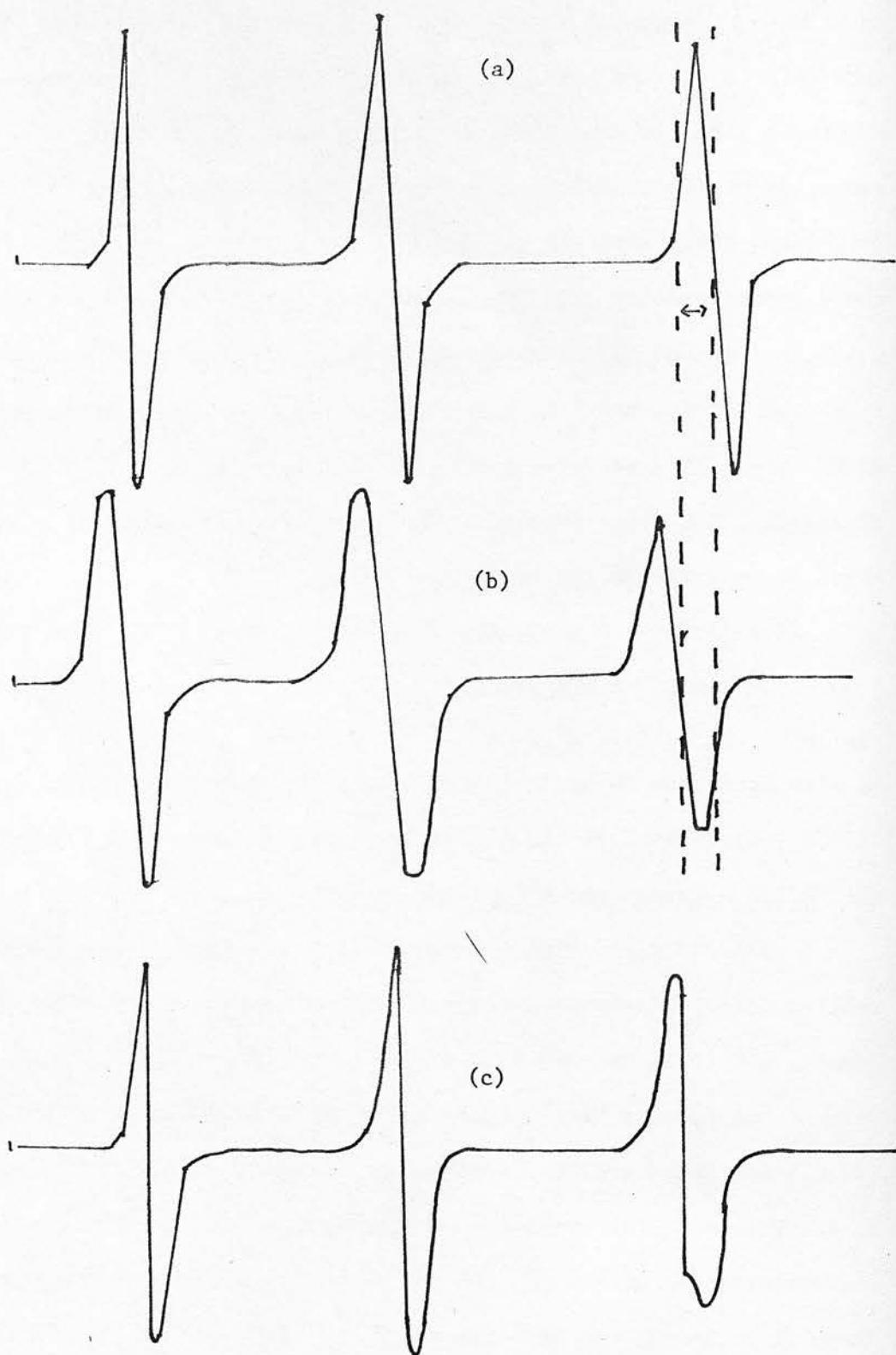


Fig. 2.7

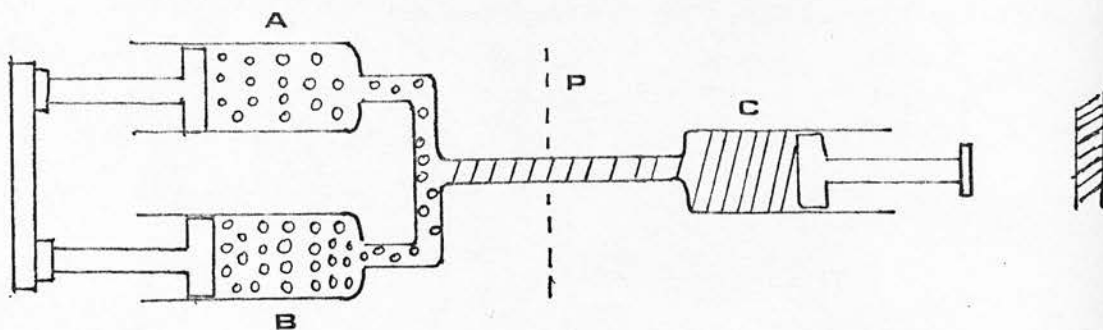


Fig. 2.7

The effect of solvent polarity on the epr spectrum of 3N8 ( $4.7 \times 10^{-4}$  M) dissolved in (a) distilled water, (b) petroleum ether and (c) in egg yolk phosphatidylcholine liposomes.

Note the alterations in peak shape and position of high field line.

Fig. 2.8



Principles of Stopped Flow

The experimental method known as stopped flow spectrophotometry is used to determine the kinetics and rates of reactions which result from the mixing of two samples, A and B. These samples are contained in two syringes which are driven by a single piston mechanism. The solutions therefore leave the two syringes with equal flow velocities and enter the mixing cell where the mixing occurs and the reaction



The mixed solution in which the reaction is now proceeding then enters a third syringe, the piston of which is driven back until it hits a stop which arrests the flow abruptly.

P marks the position of the mixing chamber.

dielectric absorption and maximising magnetic absorption. The spectra were recorded in the x-band region (3500 G) with a modulation amplitude of 1.25 G, a modulation frequency of 100 kHz, a time constant of 0.3 sec and a power input of 5 MW. The klystron was tuned at a frequency of 9.145 GHz. The spectra were obtained as a function of temperature with heating rates of about 10°C per hour controlled by a Varian E 257/WL 257 variable temperature accessory. The structure of Tempo is shown in Fig. 2.5.

The experimental design was based on the differential penetration of Tempo into lipid in the gel and liquid crystalline states. Thus the nitroxide solubility parameter, (f), can be measured from the partially resolved high field hyperfine spectral line and is approximately equal to the mole fraction of spin label in the hydrophobic region of the lipids (69) (Fig. 4.3).

(c) 3-(N-oxyl-4',4'-dimethyloxazolidine)-octane (3N8)

Experimental details were identical to those for Tempo. Fig. 2.7 shows the effect of the polarity of the medium on the spectrum and the splitting of the high field line. Fig. 2.5 shows 3N8 structure. The relative position of each spin label in the phospholipid bilayer was estimated in the following way.

(d) A solution of nickel chloride or ascorbate was added to preformed egg yolk lecithin liposomes (4 mg/20 ml) containing 50 nmoles spin label and the reduction in the spectral central line at room temperature was measured over a fixed time which varied from 10 to 20 minutes.

## 2.17 Stopped flow spectrophotometry of liposomes

Multilayered liposomes were prepared in 0.05 M KCl pH 7.2 (2 mg lipid/ml) from a lipid film containing 4 mole % dicetyl phosphate by the method described in section 2.13. The dicetyl phosphate gave the liposomes a negative charge and increased their overall diameter but did not cause an increase in salt leakage. The liposomes were introduced into one chamber of a Nortech SF3A stopped flow spectrophotometer equipped with a Datalab DL901 Transient Recorder and a recording oscilloscope. The other chamber was filled with 0.18 M KCl pH 7.2 and equal amounts of both solutions were mixed in the path of a 450 nm light source.

The initial rate of shrinking over the first second was calculated using the data collected over the first second of the reaction expressed as a fraction of the total transmittance change occurring over a 50 second period (26). As the incorporation of dicetyl phosphate has been shown to increase both the initial rate of shrinking over 1 second and the total amount of shrinking over 50 seconds, then this treatment of the data eliminated any dicetyl phosphate mediated change. Fig. 2.8 shows a diagram of the stopped flow spectrophotometer.

## 2.18 Measurement of the force area curve for mixed films of cholesterol analogues and egg yolk phosphatidylcholine

The surface pressure-area relationship was determined for monolayers spread at the air-water interface using a Langmuir trough made of PTFE. The lipids were dissolved in chloroform and injected on to the surface of a film of doubly distilled water maintained at 22.5°C. Compression of the monolayer was continuous at a rate of 4 cm min<sup>-1</sup> and a pressure of 12 dynes cm<sup>-1</sup> and the pressure curve was recorded automatically in a period no greater than 5 minutes.

## 2.19 Assay of the cholesterol side chain enzyme from adrenal mitochondria

The rats were either given (a) a 10 minute period of ether anaesthesia or (b) 10 IU ACTH (i.p.) 30 minutes prior to sacrifice. The adrenals were excised and homogenised in 0.25 M sucrose containing 10 mM Tris pH 7.4 and 0.1 mM EDTA. Mitochondria were prepared by centrifuging the homogenate at 500 x g for 15 minutes, discarding the pellet and recentrifuging the supernatant at 6,000 x g rpm for a further 15 minutes. The pellet thus obtained was once washed with buffered sucrose and centrifuged at 6,000 x g for 15 minutes. The pellet was taken up in buffered sucrose (3 ml) and resuspended by repeated mixture in a 10 ml pipette. The mitochondrial suspension (0.2 ml of 4 mg/ml) was added to an incubation mixture comprising  $\text{NADP}^+$  (20  $\mu\text{l}$  from 7.5 mg/ml,  $\text{Ca}^{2+}\text{Cl}_2$  (20  $\mu\text{l}$  from 0.05 M), cyanoketone (5  $\mu\text{l}$  from 1 mg/ml), sterol (10  $\mu\text{l}$  from 4 mg/ml) in 0.8 ml of a buffer consisting of 0.25 M sucrose (47.5 ml), IMKCl (1.0 ml), 1 M triethanolamine 0.75 ml), 1 M potassium phosphate pH 7.4 (0.5 ml), 1M  $\text{MgCl}_2$  (0.25 ml), essentially fat free BSA (50 mg) and 0.1 M EDTA (50  $\mu\text{l}$ ). The mixture was preincubated for 15 minutes at 37°C before the reaction was initiated with 5  $\mu\text{l}$  isocitrate. 0.2 ml sample was removed from each incubation after 0,2,5 and 10 minutes and added to methanol (4 ml). The amount of pregnenolone present was assayed using a previously developed radioimmunoassay.

(For column chromatography) were products of Fluka, Sharp and Dumas.  $[4\text{-}^{14}\text{C}]$  cholesterol (specific activity 30  $\mu\text{Ci}/\mu\text{mol}$ ),  $[1,2,6,7\text{-}^3\text{H}]$  cholesterol (specific activity 40-60  $\mu\text{Ci}/\mu\text{mol}$ ),  $[7\text{-}^3\text{H}]$  pregnenolone (10-15  $\mu\text{Ci}/\mu\text{mol}$ ), were purchased from The Radiochemical Centre, Amersham.

Other radioactive steroids were synthesised by Dr. R.A. Suckling in this laboratory.

## Materials

All common reagents were purchased from BDH or Sigma and were of Analar grade.

Glucose-6-phosphate, NADP, NADH, glucose-6-phosphate dehydrogenase, were obtained from Boehringer GmbH, Mannheim.

Dicetyl phosphate, cytochrome c, trypsin, horse radish peroxidase, ascorbic acid, DEAE cellulose, and blue dextran were supplied by Sigma (London).

Nonidet P42, ammonium sulphate, phenobarbitone, ammonium molybdate, coomassie blue G250, cholesterol oxidase, Triton X-100, aminophenazone, and phenol were purchased from BDH Chemicals, Poole, England.

Pregnenolone-16 $\alpha$ -carbonitrile was the generous gift of Dr. J. Babcock, Upjohn Ltd., Kalamazoo, Michigan, U.S.A.

Ethyl morphine hydrochloride was obtained from McFarlane Smith, Edinburgh.

Sephadex G75 and Sepharose 4B were supplied by Pharmacia, Uppsala, Sweden.

PPO, PPOP, L- $\alpha$ -lecithin ( $\beta$ , dipalmitoyl), L- $\alpha$ -lecithin ( $\beta$ -dimyristoyl), acrylamide, methylene-bis-acrylamide, and dihydropyran were purchased from Koch-Light, England.

Cholestyramine resin (Cuemid), Kieselgel H, and Kieselgel 60 (for column chromatography) were products of Merck, Sharp and Dohme.

[4-<sup>14</sup>C] cholesterol (specific activity 50 mCi/mmol), 1,2(n)-<sup>3</sup>H cholesterol (specific activity 40-60 mCi/mmol), [7-<sup>3</sup>H] pregnenolone (10-25 mCi/mmol), were purchased from The Radiochemical Centre, Amersham.

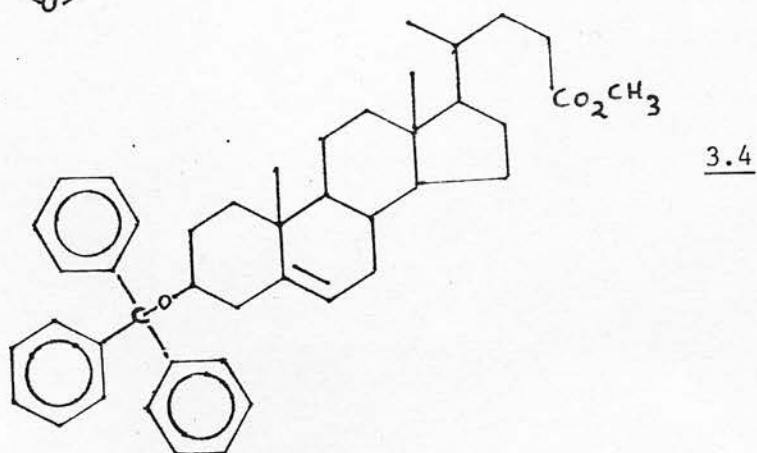
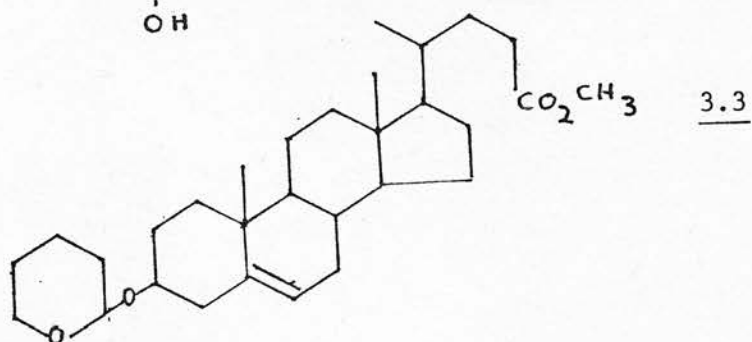
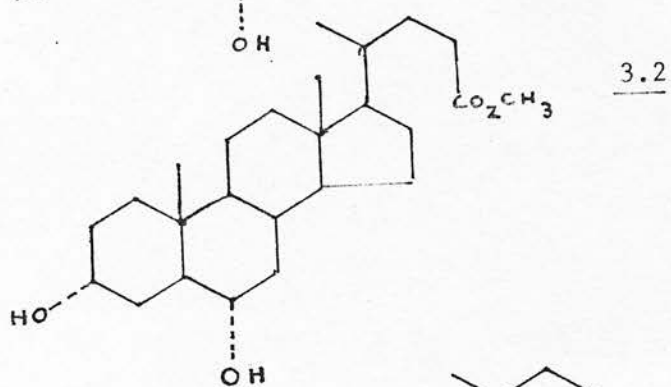
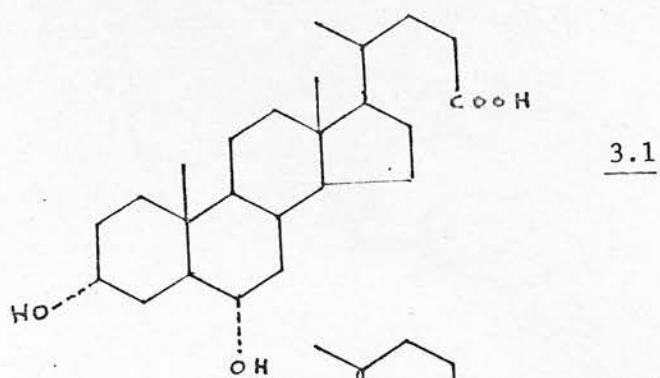
Other radioactive steroids were synthesised by Dr. K.E. Suckling in this laboratory.



3-spiro-(2'-(N-oxyl-4',4'-dimethyloxazolidine)) cholestane (3NC) was purchased from Synvar Associates, Palo Alto, California. 3B-hydroxy-26-25-(2'-(N-oxyl-4',4'-dimethyloxazolidine)) cholestane (25NC) was synthesised from 3 $\beta$ -hydroxy-26-nor-25-cholestane by Dr. K.E. Suckling.

## CHAPTER 3

### SYNTHESIS OF CHOLESTEROL ANALOGUES WITH ALTERED SIDE CHAINS



## Introduction

The synthetic scheme for the production of cholesterol analogues with altered side chains falls into 2 basic parts

- (a) Conversion of a bile acid steroid ring system to a cholestane ring system with a bile acid side chain
- (b) Extension of the five carbon unit side chain of the bile acid by one, three, four carbon atoms by the use of the appropriate Grignard reagent.

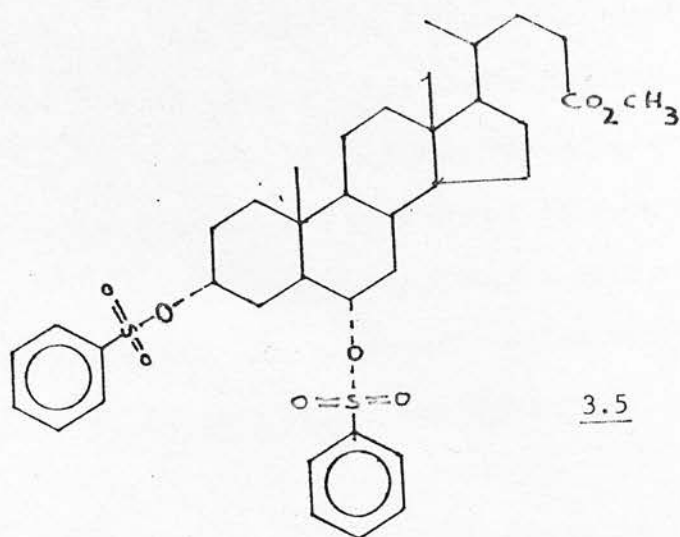
The starting material chosen for the synthetic scheme was the major swine bile acid hyodesoxycholic acid which in common with all bile acids has a cis A/B ring junction but exclusively possesses equatorial hydroxyl groups at the 3 $\alpha$  and 6 $\alpha$  positions. The hydroxyl groups are thus situated on the same side of the molecule and oriented in the correct manner to assist conversion of the A/B ring junction from a cis to a trans with a consequent inversion of configuration at the 3 $\alpha$  position.

### 3.1 Esterification of hyodesoxycholic acid

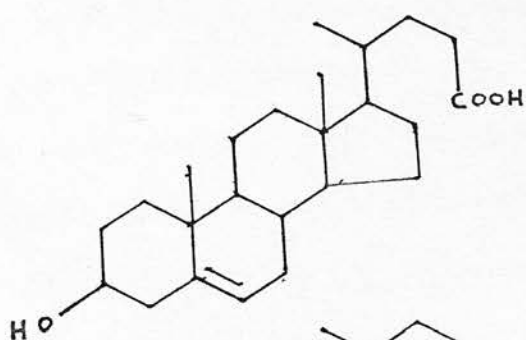
This was achieved by the use of diazomethane prepared by the method of De Boer and Backer (70) which specifically reacts with carboxylic acids leaving the alcohols untouched (71). The diazomethane was reacted with hyodesoxycholic acid (Fig. 3.1) in the presence of a small amount of methanol to enhance the reaction. The resulting methyl ester (Fig. 3.2) was formed in quantitative yield.

### 3.2 5 $\alpha$ -Cholanic acid-3 $\alpha$ ,6 $\alpha$ -diol methyl ester di-p-toluene sulphonate

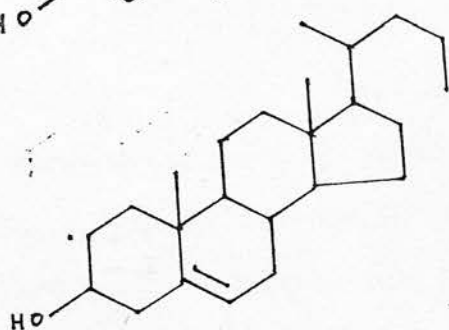
The activation of the equatorial hydroxyl groupings for elimination was readily accomplished by the reaction at 4 $^{\circ}$ C in dry pyridine containing a 2 fold molar excess of p-toluene-sulphonylchloride (72) with the hyodesoxycholic acid methyl ester (Fig. 3.2). There are three points to note in this procedure.



3.5



3.6



3.7

- (a) If wet pyridine was used the yield was very poor.
- (b) The ratio of methyl ester to pyridine was important (1 g per 4 ml gave the best yield).
- (c) The ditosylate formed by the reaction was very labile and decomposed on mild heating.

Using this method a less polar homogeneous product with an infrared spectrum identical to the methyl ester apart from the presence of an aromatic absorbance at  $1600\text{ cm}^{-1}$ , was obtained in quantitative yield.

The points raised in this section apply equally well to the preparation of tosyl esters of the various Grignard products (3.16) (3.20) (3.10) and the  $\text{C}_{24}$  hydroxy sterol (3.15).

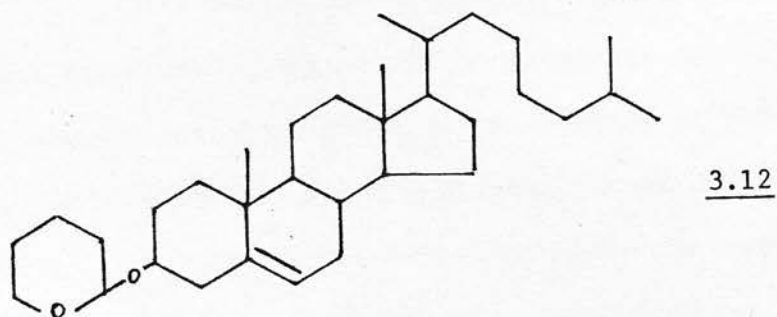
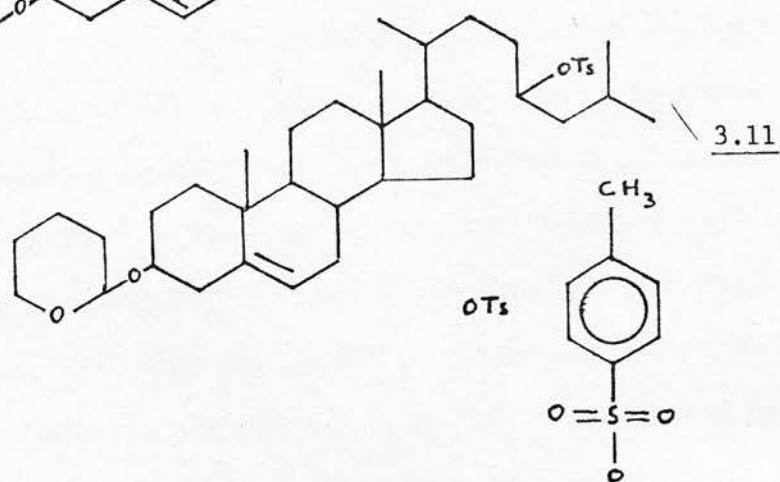
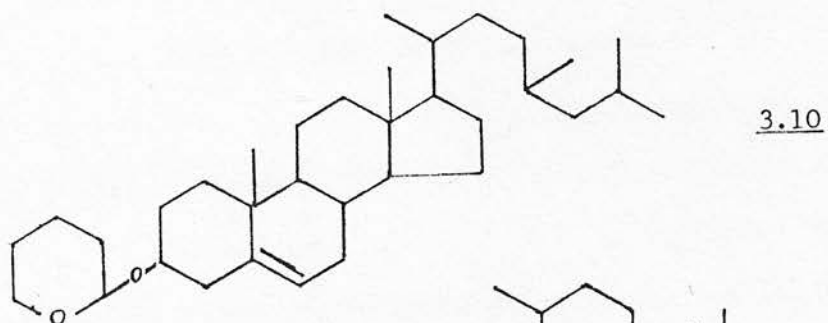
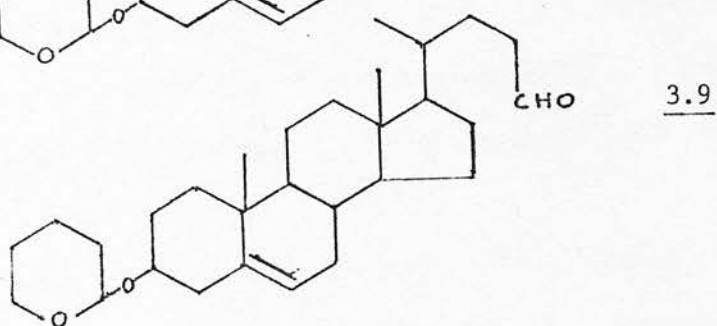
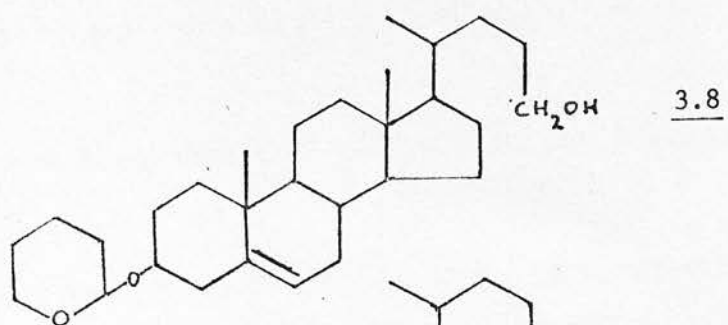
### 3.3 $3\beta$ -Hydroxy-5-cholenic acid methyl ester

The conversion of the  $3\alpha$ ,  $6\alpha$ -ditosylate to the  $3\beta$ -secondary alcohol was attempted in two ways.

(a) The ditosylate was dissolved in dimethyl formamide containing potassium acetate (73), and water and heated at  $105^\circ$  for three hours in an oil bath with constant stirring. The dimethyl formamide solution was then poured into water. Silica gel thin layer chromatography of the extracted reaction mixture showed that six products were present, of which the  $3\beta$ -hydroxy cholenic acid methyl ester when purified from the others by column chromatography was shown to be present in approximately sixteen per cent yield.

(b) In outline (b) resembles (a) in the basic solvolysis reaction (pouring dimethyl formamide into dilute acid instead of water) and included a subsequent overnight alkaline hydrolysis of the unpurified, chloroform extracted, steroidal derivatives. Purification of the reaction products by column chromatography, eluting with chloroform followed by ethyl acetate and methanol, yielded a material





which was more polar than reference characterised  $3\beta$ -hydroxy cholenic acid methyl ester. This polar product which crystallised from ethyl acetate to give a white powder (Fig. 3.6) in a yield which varied from 50-75% acid was converted to  $3\beta$ -hydroxy-5-cholenic acid methyl ester (Fig. 3.7) by treatment with diazomethane. n.m.r. analysis shows the presence of a double bond and a methyl ester.

The reason why a hydrolysis step increases the yield is explained in the paper by Bharuca et al. (73). During the dehydrosylation of  $3\alpha,6\alpha$  ditosyloxy steroids having a ring A/B cis junction, several reactions can take place.

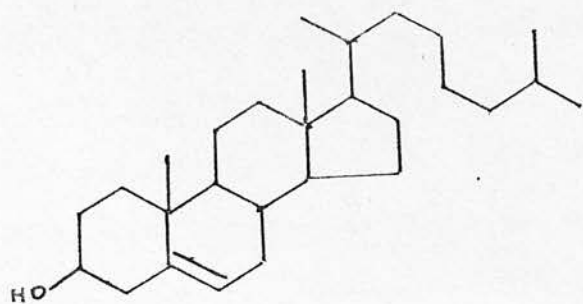
There is an  $S_N2$  mechanism operative at C-3 with complete inversion of the  $\alpha$  hydroxyl group. This is accompanied by an E1 elimination reaction in the B ring at C-6 to form the double bond. The same elimination may also occur at the C-3 instead of substitution, with the formation of a heteroannular diene impurity. The yield of the  $3\beta$ -hydroxy steroid is increased with a concomitant decrease in diene formation when the reaction is performed at an alkaline pH, which inhibits the E1 reaction as the ratio of diene to  $3\beta$ -hydroxy steroid is only determined by the relative rates of substitution and elimination processes. Alkaline hydrolysis of a heteroannular diene converts it to the corresponding  $3\beta$  hydroxy-5 $\alpha$ -compound.

### 3.4 The protection of the $3\beta$ hydroxyl grouping

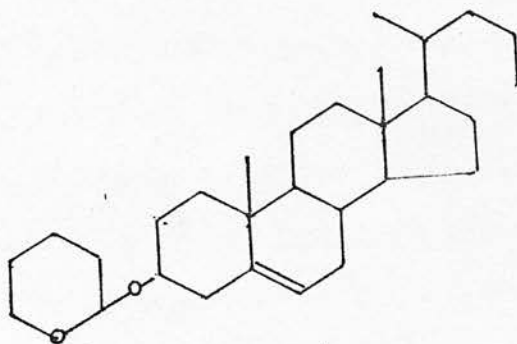
This was attempted in one of 2 ways, either by reaction with

(a) triphenylchloromethane to give  $3\beta$ -triphenyl-methoxy-5-cholenic acid methyl ester (Fig. 3.4) or (b) dihydropyran yielding  $3\beta$ -tetrahydropyranyloxy-5-cholenic acid methyl ester (3.3).

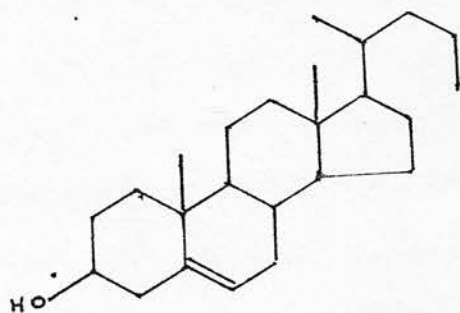
(a) Helferich et al. (74) showed that primary and secondary alcohols can be converted into their respective trityl ethers by reaction with triphenylchloromethane in pyridine. Thus equimolar quantities of the



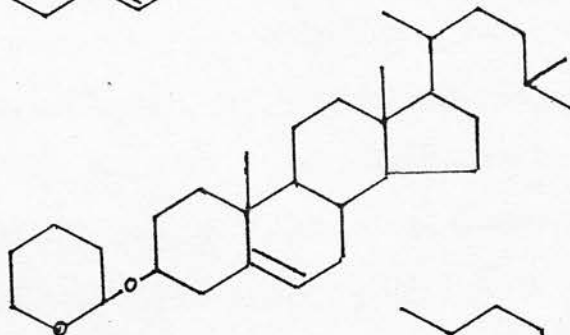
3.13



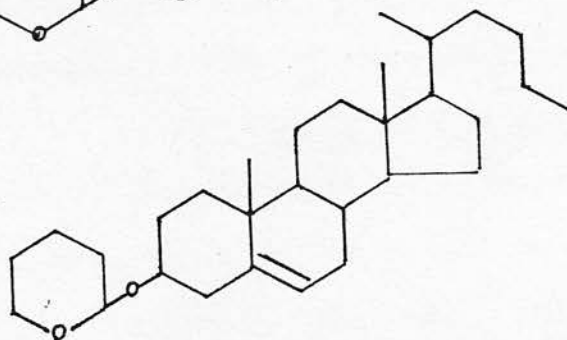
3.14



3.15



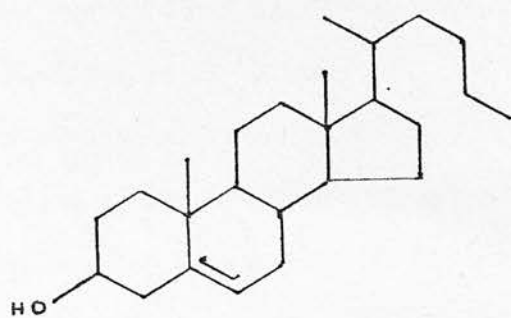
3.16



3.17

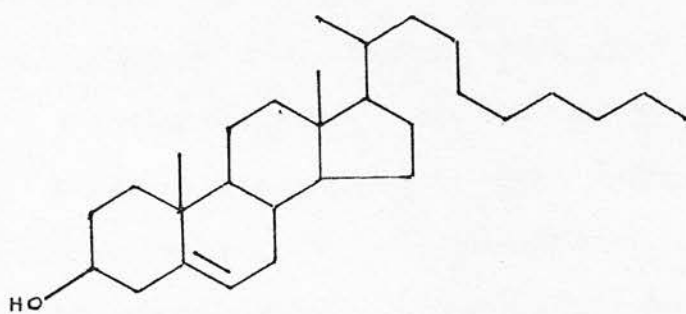
3 $\beta$ -hydroxy-5-cholenic acid methyl ester and triphenylchloromethane, previously purified by recrystallisation in the presence of acetyl chloride (72), were reacted in dry apparatus in an oil bath at 110°C in the presence of catalytic amounts of phosphorous oxychloride. Any trace of water results in the liberation of hydrochloric acid which would cleave any adduct formed. The products were extracted with dichloromethane and isolated by column chromatography. Due to difficulty in separating the protected methyl ester from the impurity triphenylhydroxymethane the yield was very variable but was less than 47% (b) 2-3 dihydropyran is a reagent which reacts under mild acid catalysis with primary and secondary alcohols to form tetrahydropyranyl ethers which are stable to bases, Grignard reagents, lithium aluminium hydride, and to oxidation (72). Due to its high reactivity with carboxylic acids, it was essential that the acid at C<sub>24</sub> was esterified prior to its exposure to dihydropyran.

The method used was based on those developed by Greenhalgh et al. (75) and Dasgupta et al. (76) for the protection of cholesterol and 3 $\beta$ -hydroxy-24-nor-cholen-23-al respectively and is very clean as judged by TLC. The tetrahydropyranyl ether was formed by reaction of the secondary alcohol with a 3 fold molar excess of freshly distilled dihydropyran in dry chloroform in the presence of catalytic amounts of phosphorus oxychloride. The apparatus and chemicals must be dry because the presence of water can cause the liberation of hydrogen chloride which can cleave any adduct formed. The reaction was allowed to proceed overnight and the protected product isolated by silica gel column chromatography on elution with ethyl acetate:chloroform (5:95 v/v). The yield of this reaction was 70% as compared to the slightly higher figures of 80% reported.

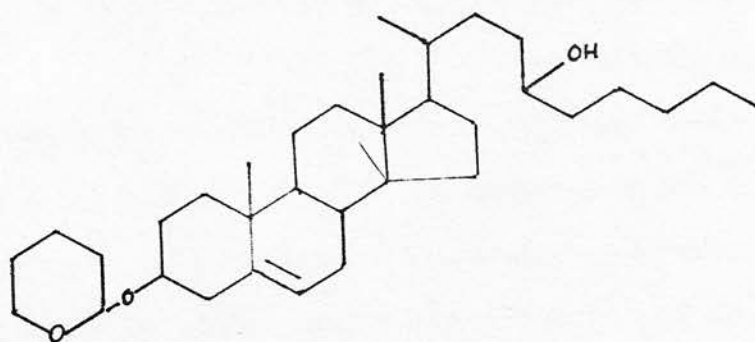


3.18

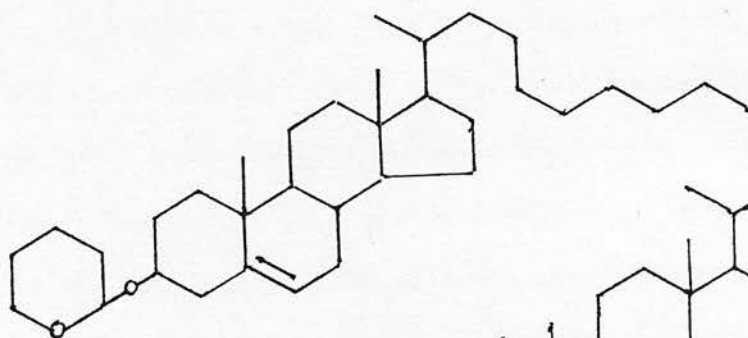
C<sub>25</sub>



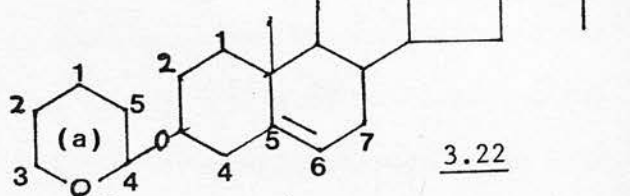
3.19



3.20



3.21



3.22

### 3.5 Lithium aluminium hydride reduction

There <sup>are</sup> two steps in the synthetic scheme that involve the use of lithium aluminium hydride.

(a) Conversion of the carboxylate methyl ester grouping to a primary alcohol (3 $\beta$ -2'-tetrahydropyranyloxy-5-cholen-24 ol) (Fig. 3.2).

This is a well characterised reaction involving the addition of a large excess (2-4 mole ratio) of lithium aluminium hydride to a solution of dry methyl ester. The reduction of esters is described as an initial nucleophilic displacement which in effect amounts to an addition-elimination step followed by a reduction step.

After a four hour reflux in dry apparatus fitted with a calcium chloride guard tube the excess reagent was destroyed and the product was isolated by column chromatography. The yield obtained by this procedure is 70% (85% in (72)) IR analysis cannot detect the presence of a carboxyl group consistent with its conversion to an alcohol.

(b) The second reaction in the synthetic scheme that required the use of lithium aluminium hydride was the reduction of a primary p-toluene-sulphonyl-ester to the corresponding methyl compound.

This is common to the synthesis of the protected C<sub>28</sub> (Fig. 3.12).

C<sub>25</sub> (Fig. 3.17), C<sub>24</sub> (Fig. 3.14) and C<sub>29</sub> (Fig. 3.21), and is readily accomplished with a quoted median yield of 65% (72).

The actual conditions for the conversion were identical to those described above and the reaction proceeded in approximately 50-60% yield (90% in (72)).

### 3.6 Production of 5-cholen-3 $\beta$ -ol

The synthesis of 5-cholen-3 $\beta$ -ol was accomplished using 3-2'-tetrahydropyranyloxy-5-cholen-24-ol as starting material.

The synthetic scheme involved (a) an initial activation of the



primary alcohol on  $C_{24}$  by reaction with p-toluene sulphonyl chloride followed by (b) a lithium aluminium hydride mediated reduction. 5-cholen-3 $\beta$ -ol was then easily prepared by (c) acidic cleavage of the 3 $\beta$ tetrahydropyranyl ether.

(a) The p-toluene sulphonyl ester was prepared by reaction of the free alcohol with an excess of p-toluene sulphonyl chloride following the method previously described in this chapter.

Infra red analysis of the concentrated reaction mixture detected the presence of an aromatic ring and thin layer chromatography indicated that a single, less polar, product had been formed.

(b) This procedure has already been described in detail in a separate section. Crystalline 3 $\beta$ tetrahydropyranyl ether was obtained from the acetone solution of a Keiselgel 60 column eluate. Infra red analysis showed that the aromatic group had been removed and mass spectrometry gave the major peak at 328. This was consistent with loss of the tetrahydropyran and the sterol 3 $\beta$  hydroxyl group from the desired compound.

(c) Acid hydrolysis of 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholene yielded 5-cholen-3 $\beta$ -ol as needle like crystals from methanol with a molecular ion of 344.

### 3.7 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholen-24-ol (Fig. 3.9)

The foregoing alcohol was oxidised to an aldehyde in one of two following ways

- (1) By oxidation with chromium trioxide pyridine complex (77) and
- (2) By reaction with a silver oxide:celite (78).

(1) After reaction with the Collins reagent (77), TLC of the crude material showed that four products had been formed, one of which was separated in 25% yield.

Using this procedure Collins obtained cholestenone from cholesterol in 64% yield, however there are two problems associated with this procedure.

- (a) The oxidation will proceed beyond the aldehyde with formation of a carboxylic acid impurity.
- (b) As it is an acidic reagent it tends to cleave the 3 $\beta$  tetrahydropyranyl ether linkage.
- (2) Essentially this method is that of (78) and involved the preparation of a silver oxide:celite reagent formed by the combination of silver nitrate (0.117 M), potassium carbonate (2 mmoles), and celite (1.5 g). This gave sufficient oxidation complex to react with 1 mmole of alcohol during an overnight reflux. The aldehyde formed was easily isolated by column chromatography due to the large difference in polarity of starting material and product was found to be present in greater amounts (40-70% yield as compared to 25% with (1)).

The silver oxide gave a much cleaner conversion to product and proceeds to completion with no further conversion of the aldehyde.

In both (a) and (b) the appearance of a sharp peak in the IR trace at  $1707\text{ cm}^{-1}$  was indicative of the presence of a carboxyl group. In common with all tetrahydropyranyl ethers no molecular ion was detected, but assuming that the molecular weight was 442, the disintegration pattern was explained in terms of removal of dihydropyran and a hydroxyl group ( $M^+ - 102$ ). The major peak has a molecular weight of 340.

### 3.8 Extension of the bile acid five carbon side chain by addition of the appropriate Grignard reagent

The synthesis of (3.10), (3.20) and (3.16) were achieved using 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholen-24-ol and a Grignard prepared from purified 1-bromo-2-methyl propane, 1-iodo-pentane, and iodomethane respectively. The method employed was similar in all cases and was based on those of Petrov and Stuart-Webb (79) and

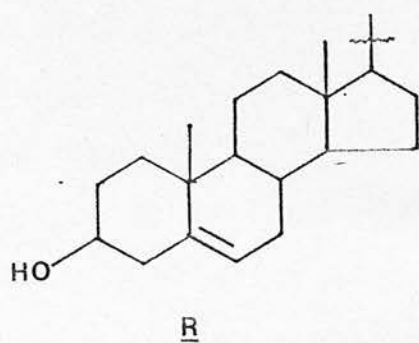
Dasgupta et al. (76). The alcohol formed from 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholesterol was isolated by column chromatography of the concentrated reaction mixture on Kieselgel 60 prepared in dichloromethane and eluted with 5% ethyl acetate:95% chloroform. Using this procedure the yield of product was 60-70% which is comparable with the 80% of Dasgupta et al. (76) and 45% of Petrov (79).

The procedures required for the activation of the secondary alcohol at C<sub>24</sub> and its subsequent reduction by lithium aluminium hydride have already been mentioned.

### 3.9 Synthesis of 26,27-dinor-cholestane (3.18), 26,27-dinor-25-isopropyl-cholest-5-en-3 $\beta$ -ol (3.13), and 26,27-dinor-25-butyl-cholest-5-en-3 $\beta$ -ol (3.19)

The final step in the synthetic scheme was the removal of the tetrahydropyranyl group at the 3 $\beta$  position to give the free secondary steroid alcohols (C<sub>25</sub>, C<sub>28</sub>, and C<sub>29</sub> respectively). The method used was essentially a combination of those of Dasgupta (76) and Greenhalgh et al. (75) and involved the addition of ethanol containing a few drops of concentrated hydrochloric acid to a suspension of the compound in ethanol. Warming to 50°C gave a clear solution from which a crystalline, unprotected, product was obtained by evaporation of the ethanol under a slow stream of nitrogen. It was cooled, filtered, and recrystallised from methanol giving an average 65% yield of product which had thin layer chromatographic properties and infra red spectra similar to those of cholesterol.

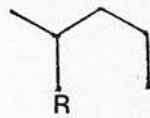
As a final check of their side chain lengths the analogues were injected as the free sterol, on to a column of 100-120 mesh gas chrom Q with 1 per cent SE 30 adsorbed to it. The G.L.C. column oven was maintained at 238°C and attached to a flame ionisation detector. The retention times of the cholesterol analogues were noted



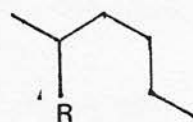
C<sub>19</sub>



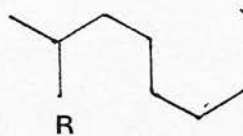
C<sub>21</sub>



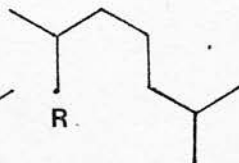
C<sub>24</sub>



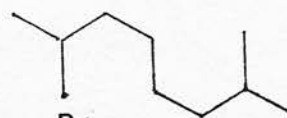
C<sub>25</sub>



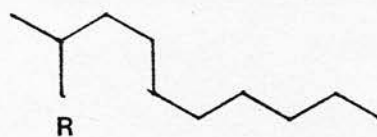
C<sub>26</sub>



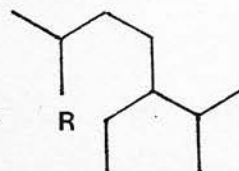
C<sub>27</sub>



C<sub>28</sub>



C<sub>29</sub>



Sitosterol

Fig. 3.23

and a plot of the log of each sterol's retention time relative to that of cholesterol, which was given the value (1), against the number of carbon atoms in each analogue was constructed. Using this hydrophobic column material it has been shown to be possible to separate a mixture of fatty acids according to their chain lengths. The absolute retention time of each fatty acid varied in direct proportion to the number of carbon atoms in the chain. In the same way the side chain analogues possessed relative retention times which were consistent with proposed side chain structure.

The  $C_{19}$ ,  $C_{21}$  and  $C_{26}$  (Fig. 3.23) previously prepared by Dr. Keith Suckling were included to give a better estimate of the graph. Fig. 3.24 shows the calibration curve.

Thus the sequential elution from a G.L.C. column taken in combination with the other properties of the intermediates and final products indicates that the synthetic scheme described is capable of producing a set of cholesterol side chain analogues based on the 5 carbon unit of the bile acid side chain.

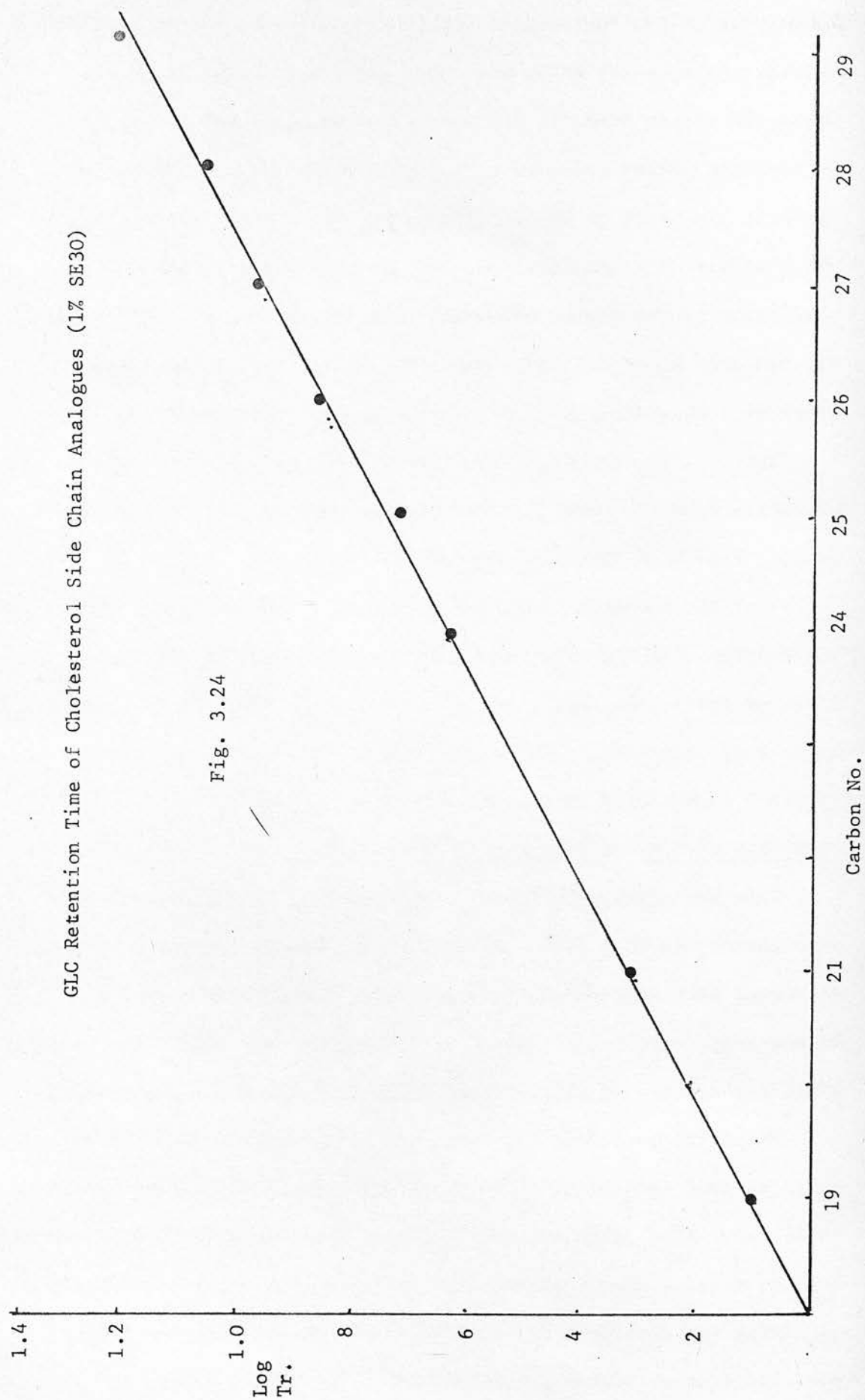
### 3.10 Synthesis of $\{7-^3H\}$ -7 $\alpha$ hydroxycholesterol

This was achieved using the procedures of Malinow et al. (80) and Johnson and Lack (81). Although the starting materials were different both methods used tritium labelled  $NaBH_4$  to reduce a keto group at the 7 position in the steroid ring system. The reduction yielded a mixture of sterols hydroxylated at the  $\beta$  and  $\alpha$  positions.

Using the method of Malinow, 7-keto-cholesterol (0.02 mole) was dissolved in 9 ml of methanol, 5 ml of freshly prepared ethanolic tritiated.  $NaBH_4$  (100 mCi of 156 mCi/mg specific activity) was added, and the solution was incubated for 3 days at  $4^\circ C$ . Any borohydride remaining was destroyed by the addition of water (8 ml) and the steroids extracted with petroleum ether (2 x 30 ml,  $60^\circ C$ ).

GLC Retention Time of Cholesterol Side Chain Analogues (1% SE30)

Fig. 3.24





The extracts were combined and evaporated to dryness under nitrogen. To ensure that all remaining tritium was stably linked to C<sub>7</sub> the steroid residue was dissolved in methanol (5 ml). The solvent was distilled, collected by evaporation and assayed for tritium in a liquid scintillation spectrometer. The procedure was repeated three times until negligible radioactivity was present in the distillate. The 7 $\beta$ - and 7 $\alpha$ -hydroxycholesterol isomers in the residue were isolated by TLC on (20 x 20 x 0.2 cm) silica gel plates with authentic standards in adjacent lanes. Ethyl acetate:benzene (13.7 v/v) was used as the developing solvent and the radioactive peaks correspond with those of the authentic standards. The reaction was very inefficient in my hands, with a recovery of 1 per cent of added radioactivity. 7 $\alpha$ -Hydroxycholesterol accounted for a quarter of the recovered material the remaining threequarters being 7 $\beta$  hydroxycholesterol.

### 3.11 Synthesis of 7 $\alpha$ -hydroxycholesterol (82)

Recrystallised cholesterol (300 mg) was dissolved in chloroform containing haematoporphyrin methyl ester (1 mg) and placed in a photo-oxygenation chamber. Oxygen was bubbled through the solution during the subsequent eighteen hour incubation and the progress of the reaction was monitored by TLC. Phosphomolybdic acid spray revealed that the blue cholesterol spot disappeared and a turquoise spot corresponding to cholesterol-7-hydroperoxide appeared in its place.

The hydroperoxide was transformed to 7 $\alpha$ -hydroxycholesterol by a 45 minute reduction with potassium iodide (1.5 g) in a refluxing solution of ether; ethanol (1:5 v/v) containing 4 drops of 10% acetic acid. The solvent was evaporated and the excess iodine was removed by washing with sodium thiosulphate. 7 $\alpha$ -Hydroxycholesterol was separated by thin layer chromatography on a silica gel (20 x 20 x 0.2 cm)

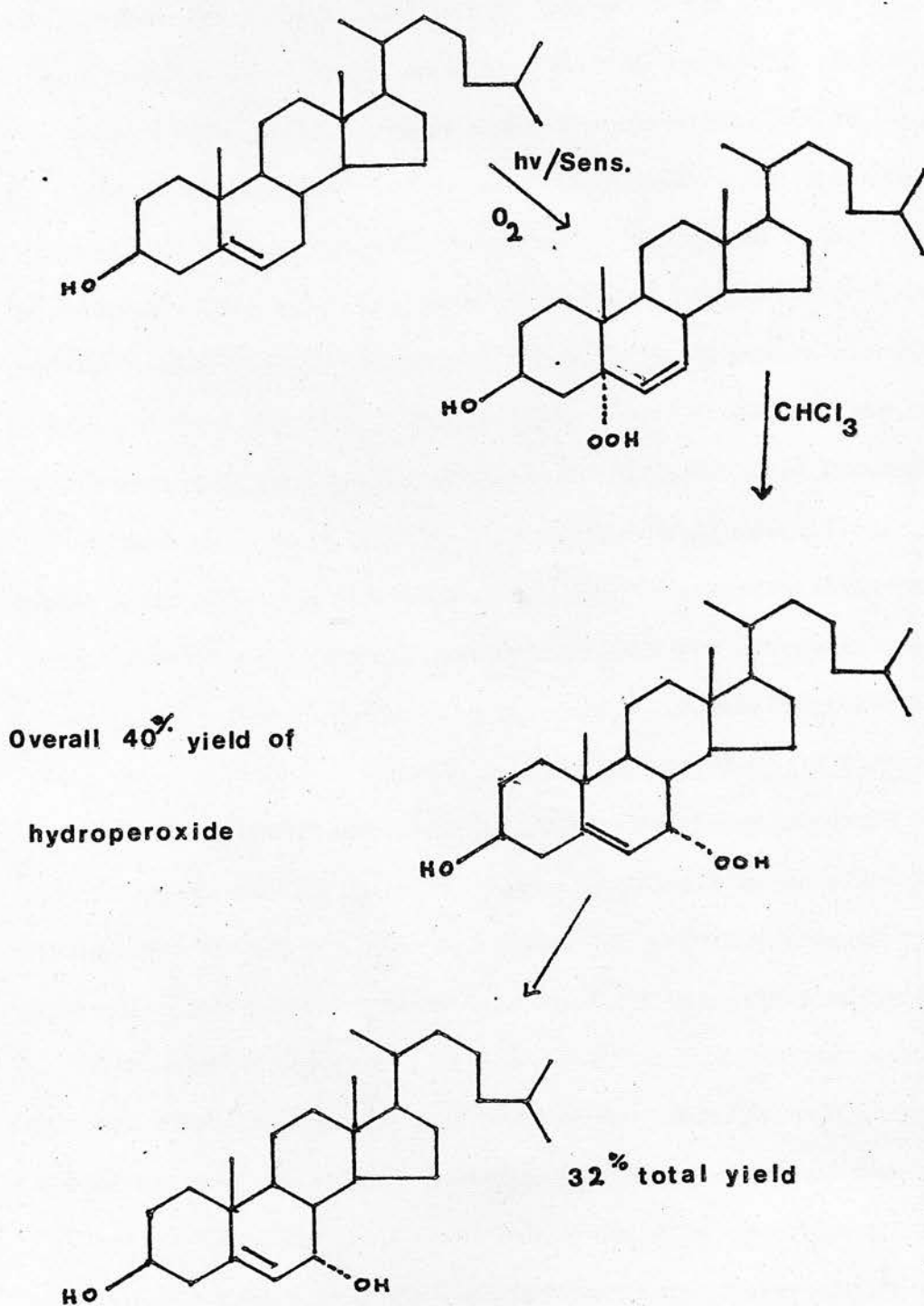


Fig. 3.25

Synthetic route for production of 7 $\alpha$  hydroxycholesterol.

plate developed with benzene; ethyl acetate (7:13 v/v) by comparison with standard 7 $\alpha$ -hydroxycholesterol. The two step reaction produced white crystals from methanol (80 mg; 27% yield compared to a literature value of 32%).

Fig. 3.25 describes the synthetic route.

### 3.12 Experimental

#### General directions

Organic extracts were dried over anhydrous sodium sulphate unless otherwise stated. Solutions were evaporated under reduced pressure using a rotary evaporator. The purity of all compounds was established using thin layer chromatography (TLC) on Cam. Lab. POLYGRAM SILG/UV 254. precoated plates. Radiochemical purity of all radioactive products was monitored using a Panax thin layer scanner R.T.L.S.(l.A.). Analytical samples were dried in vacuo prior to analysis. Melting points, which are uncorrected, were determined on a Kofler Micro Hot Stage. Infra-red spectra (I.R.) were recorded on a Perkin Elmer 257 grating IR spectrometer using chloroform solutions. Frequencies of IR peaks are quoted in  $\text{cm}^{-1}$ .

Nuclear magnetic resonance spectra (n.m.r.) were obtained by Mr. John Miller of the University of Edinburgh Chemistry Department on a Varian HA 100 or XA 100. Chemical shifts are quoted in  $\delta$  values and the following abbreviations are used.

s = singlet      d = doublet      t = triplet  
q = quartet      m = multiplet

Fig. 3.22 describes the numbering system adopted for H resonances.

Gas liquid chromatography (GLC) was carried out using a Pye Unicam Series 100 GLC fitted with a 1% SE 30 on 100/120 Gas Chrom. Q column and using a flame ionisation detector. Mass spectra were obtained by Dr. Norman Chalmers of the University of Edinburgh

Department of Pharmacology on a LKB 9000 MGSS spectrometer using trimethylsilyl derivatives of the compounds, except in case of tetrahydropyran ethers, and a 1.5% SE 30 column. The organic extracts were purified by chromatography on Merck Keisegel 60.

#### Hydodesoxycholic acid methyl ester

Diazomethane (1 g) was prepared by the dropwise addition of a solution of N-methyl-N-nitrosotoluene-4-sulphonamide (7.0 g) in anhydrous ether (100 ml sodium dried) to an ethanolic solution (30 ml) containing potassium hydroxide (1.6 g) and a few drops of water to enhance the solution of potassium hydroxide. The ethanol was heated to at least 50°C and the diazomethane evolved collected over a half hour period by condensation of the yellow etherate. The closed collecting vessel was surrounded by an ice jacket and fitted with an outlet tube which was immersed in a further open conical flask containing dry ether to collect any escaped diazomethane. After all the N-methyl-N-nitrosotoluene-4-sulphonamide solution was added, the procedure was repeated with a further 50 ml of dry ether in order to ensure that no diazomethane remained in the apparatus. The solutions in the main and secondary collecting flasks were combined and stored in ice.

Hydodesoxycholic acid (9 g) was added to anhydrous ether (400 ml) containing methanol (5 ml) to enhance (a) the methylation reaction and (b) the solubility of hydodesoxycholic acid, and placed in ice. The diazomethane was added in 10 ml portions and the solution swirled. After all the diazomethane had been added the mixture was then tested for the presence of excess diazomethane by acetic acid in ethanol (1:1).

The resulting methylated steroid was obtained by evaporation of the solvent yielding a light yellow oil which formed 11.4 g of a yellowish-white powder after drying under vacuo (100% yield). No further purification was required as TLC showed one product. IR C=O 1720, vC-OH. 3400, vC=H 2850.

5 $\alpha$ -Cholanic acid-3 $\alpha$ ,6 $\alpha$ -diol methyl ester-di-p-toluene sulphonate

5 $\alpha$ -Cholanic acid-3 $\alpha$ ,6 $\alpha$ -diol methyl ester (11.4 g) was dissolved in dry pyridine (44 ml, dried by storing over a molecular sieve) and placed in ice. To this solution was added, gradually with swirling, p-toluene sulphonyl chloride (12 g) and the resulting solution placed at 4°C for 18 hours during which time colourless needles of pyridine hydrochloride were formed and the solution became yellow. The mixture was then poured into an ice cold solution of dilute hydrochloric acid (100 ml) and either a white crystalline mass or a yellow oil was deposited on the ice. In both cases the acidic solution was extracted with dichloromethane (3 x 50 ml) and ~~the~~ <sup>evaporated</sup> the excess solvent was by an initial rotary evaporation step at room temperature.

(b) Residual solvent was removed by a stream of nitrogen and addition of methanol to the residue.

(c) The methanol was removed by a further exposure to the nitrogen, and evaporation under vacuo using an oil pump.

A caramel gum (15 g) resulted from the procedure, TLC of which in dichloromethane showed that only one product was present. No further purification due to lability of tosylates.

IR vC2O 1720, v aromatic 1600, and vC-H 2850.



### 3 $\beta$ -Hydroxy cholenic acid

5 $\alpha$ -Cholanic acid-3 $\alpha$ ,6 $\alpha$ -diol methyl ester di-*p*-toluene sulphonate (15 g) was placed in a conical flask in an oil bath, which was in turn placed on a magnetic stirrer hot plate. Dimethylformamide (120 ml), water (12 ml), and potassium acetate (25 g), were added and the solution heated to 105°C with constant stirring. After 4 hours the mixture was poured into dilute hydrochloric acid without cooling and the resulting yellow-white precipitate was filtered, washed with water, and immediately saponified by refluxing overnight with 4% (w/v) methanolic potassium hydroxide. The yellow liquid was cooled and poured into dilute hydrochloric acid once again. The white water-saturated precipitate was filtered and extracted with chloroform:methanol (2:1).

In addition the same extraction procedure was carried out on the gum formed on the sides of the flask after acidification and the 2 extracts combined and concentrated by rotary evaporation. Recrystallising the material from ethyl acetate yielding 9 g of a white powder. TLC showed it contained one major product and two minor less polar contaminants (64% yield).

### 3 $\beta$ -Hydroxy-cholenic acid methyl ester

The foregoing 3 $\beta$ -hydroxy cholenic acid (9 g) was dissolved in dry ether (400 ml) containing methanol (5 ml) and reacted with diazomethane (1 g) in ether solution. The reaction mixture was concentrated by rotary evaporation and dried under vacuo using an oil pump. The contaminating upper two spots were removed by chromatography on Kieselgel 60 (270 g) prepared in dichloromethane. Sequential elution with dichloromethane, dichloromethane:chloroform (1:1), and chloroform gave the pure 3 $\beta$ -hydroxy cholenic acid methyl ester which crystallised from methanol to give a white powder (6.92 g)



78% from ditosylate mp  $144^{\circ}\text{C}$  (lit  $145\text{--}146^{\circ}\text{C}$ ) IR  $\nu$  C=O  $1720$ ,  $\nu$  C-H  $2930$ .  
 n.m.r.  $3.64\ \delta$  ( $\text{CO}_2\text{CH}_3$  at  $\text{C}_{24}$ ),  $5.33\ \delta$   $\text{C}_5$  attached to  $\Delta^5$  double bond.  
 M.S.  $\text{M}^+$  461.

### $3\beta$ -Triphenylmethoxy-5-cholenic acid methyl ester

$3\beta$ -Hydroxy-5-cholenic acid methyl ester (2.5 g) was dissolved in dry pyridine (6 ml) and mixed with triphenylchloromethane (purified by recrystallisation from benzene containing 20% acetyl chloride and by washing with light petrol containing acetyl chloride). The reaction was initiated by heating the mixture on an oil bath for 10 hrs at  $110^{\circ}\text{C}$ . After cooling the pyridine was removed by rotary evaporation, water was added to the resulting oil and the mixture was heated to convert the triphenylchloromethane to triphenylhydroxymethane. The solution was cooled, extracted with dichloromethane (3 x 30 ml), and concentrated by rotary evaporation. The mixture was chromatographed on Kieselgel 60 (70 g) made up in dichloromethane, and elution with the same solvent removed the non-polar product and a portion of the triphenylhydroxymethane which proved impossible to separate from the product using chromatographic techniques and differing elution systems (such as petroleum ether:dichloromethane 100:0 to 0:100) 1.5 g of triphenylmethane derivative:triphenyl hydroxymethane mixture <47% yield.

$\nu$  C-H  $2900\ \text{cm}^{-1}$ ;  $\nu$  C=O  $1720\ \text{cm}^{-1}$ ,  $\nu$  C-C (aromatic)  $1600\ \text{cm}^{-1}$ .

### $3\beta$ -2'-tetrahydropyranyloxy-5-cholenic acid methyl ester

$3\beta$ -Hydroxy-5-cholenic acid methyl ester (6.92 g) was dissolved in dry chloroform (20 ml dried by passage down a column of roasted Kieselgel H and subsequently over sodium sulphate) and placed in a dried flask with a calcium chloride guard tube. Freshly distilled dihydropyran (6 ml) and 10 drops of phosphorus oxychloride were added, the guard tube was replaced and the yellow solution was left

overnight at room temperature. The mixture became dark green during the reaction and the coupling was stopped by dilution with dry chloroform and addition of chilled sodium bicarbonate. The chloroform layer was separated, the water layer extracted with chloroform (2 x 20 ml) and the extracts concentrated to give a reddish brown oil which was purified by column chromatography on Kieselgel 60 (200 g). The column was prepared in  $\text{CH}_2\text{Cl}_2$ , and eluted with 5% ethyl acetate; <sup>gave a</sup> 95% chloroform purified product (which crystallises from methanol from more polar impurities).

5.62 g (70% yield) m.p. 110-111°C.

IR  $\nu_{\text{C-H}}$  2880, 2440, 2620, 2710;  $\nu_{\text{C=O}}$  1720.

n.m.r. 5.3t(C=C), 4.62<sub>s</sub> (2), 3.92<sub>q</sub> (6a, 3).

3.65 ( $\text{CO}_2\text{CH}_3$  at 24) 1 m (saturated alkyl).

M.S.  $\text{M}^+$  472.

#### 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholen-24-ol

The foregoing 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholenic acid methyl ester (5.6 g) was dissolved in tetrahydrofuran (25 ml) which had been freshly distilled into dry glassware from a solution of tetrahydrofuran containing a few milligrams of lithium aluminium hydride to minimise the formation of hydroperoxides. Lithium aluminium hydride (450 mg) was then added and the flask fitted with a condenser topped by a calcium chloride guard tube. The reaction proceeded during a 4 hour reflux after which the catalyst was destroyed by the sequential addition of water (0.45 ml) followed by 15% sodium hydroxide (0.45 ml) and finally a further amount of water (1.35 ml). A light grey complex was formed by this process, and this was placed in a filter funnel and washed with chloroform (100 ml) to remove any adhering steroid. The complex was discarded and the chloroform extract concentrated, dried under vacuum and applied to a

column of Kieselgel 60 (180 g) made up in dichloromethane. When the column was eluted with chloroform and then 5% ethyl acetate:95% chloroform a colourless oil was formed which on leaving overnight was transformed into a white opaque solid which crystallised into transparent needle like crystals from ethyl acetate. 4.0 g 78% yield. m.p. 134-135°C.

IR  $\nu$ C-OH 3400,  $\nu$ C-H 2860, 2440, 2620, 2710.

n.m.r. 5.3 d (C=C), 4.7 s (H at 2a), 3.9 q ( $\begin{smallmatrix} 2 \text{ H at } 6a \\ \text{H at } 3 \end{smallmatrix}$ ), 3.55 d ( $\begin{smallmatrix} \text{CH}_2\text{OH} \\ \text{at } \text{C}_{24} \end{smallmatrix}$ ), 9 t (saturated alyl), 4.9 s (OH at C<sub>24</sub>).

M.S. M-102 = 414.

### 3 $\beta$ -2'-tetrahydropyranyloxy-cholen-24-ol

The oxidising reagent used in this reaction was prepared by rapidly mixing acid washed celite (3.0 g) with an aqueous silver nitrate solution (2 g in 100 ml) and potassium carbonate (0.8 g) for 15 minutes. The mixture was filtered, sucked dry in air on a Buchner flask, and further dried in a vacuum desiccator for 5 hours. The yellow filter cake was then transferred to a round bottomed flask containing dry benzene, 10 ml of which was evaporated off to ensure the complete absence of water. The 3 $\beta$ -2'-tetrahydropyranyloxy-cholen-24-ol (1 g) in dry benzene was mixed with the oxidising reagent and <sup>the</sup> solution was refluxed overnight using a condenser with a calcium chloride guard tube, cooled, and filtered through celite. The resulting yellow solution was evaporated and purified by column chromatography on Kieselgel 60 (30 g) prepared in dichloromethane. Elution with 5% ethyl acetate:95% chloroform yielded white needles from ethyl acetate solution. The yield of the aldehyde seemed very variable from preparation to preparation and is probably due to the presence of water in the oxidising agent. m.p. 125-126°C.

IR  $\nu_{\text{C-H}}$  2860;  $\nu_{\text{C=O}}$  1707.

M.S. No molecular ion  $M^+-\text{THP} = M-102 = 340$ .

Calculated molecular weight = 442.

26,27-Dinor-25-isopropyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-ol

In order to prepare the appropriate Grignard reagent for the desired side chain analogue of cholesterol, dry ether washed magnesium turnings (240 mg, 10 mmole) were added to a crystal of iodine in a dry round bottomed flask. This was gently warmed until iodine vapour filled it. The flask was cooled and fitted with a condenser, a  $\text{CaCl}_2$  guard tube, and an addition funnel. The magnesium was then covered with dry ether (5 ml) and the addition funnel was filled with dry ether (5 ml) and freshly distilled 1-bromo-2-methylpropane (2 ml, 18 mmoles). 1 ml of the mixture was introduced into the reaction flask whose contents were magnetically stirred. The remainder of the halide was added dropwise over a ninety minute period with constant stirring and this was followed by a 30 min. reflux of the mixture to destroy any residual magnesium.

The 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholen-24-ol (0.45 g) was dissolved in dry benzene (15 ml) and added to the excess of Grignard and the mixture was heated to 78°C with the condenser switched off to remove the ether. The benzene remaining was refluxed overnight with the condenser fitted with a calcium chloride guard tube, after which it was cooled and the Grignard complex was decomposed by the addition of saturated ammonium chloride (3.8 g in 10 ml). The Grignard product was isolated by ether extraction of the aqueous layer, and subsequent evaporation of the ether. The purified product was eluted by 95% chloroform:5% ethyl acetate from a Kieselgel 60 column (13 g) made up in dichloromethane.

300 mg product 70% yield.

IR  $\nu_{\text{C-OH}}$  3400;  $\nu_{\text{C-H}}$  2840, 2440, 2620, 2710.

n.m.r. 9 m (saturated alkyl), 3.54 s (OH at 24), 5.3 ( $H_5$  attached to C=C).

26,27-Dinor-25-isopropyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-p-toluene sulphonate

The 26,27-dinor-25-isopropyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-p-toluene sulphonate was prepared in a similar manner to the 5-cholenic acid 3 $\alpha$ ,6 $\alpha$ -diol methyl ester di-p-toluene sulphonate. 26,27-dinor-25-isopropyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-ol was dissolved in dry pyridine (1.2 ml and p-toluene sulphonyl chloride (300 mg) added as before. After extraction of the ditosylate the chloroform was removed by a nitrogen stream and vacuum desiccation. The purity of the tosylate was checked by TLC and the one spot material utilised in the subsequent reduction step without any further purification.

IR  $\nu$ C-H 2920;  $\nu$ C-C 1580

C-H deformation 1420, 1350.

26,27-dinor-25-isopropyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-ene

The reduction of the tosylate with lithium aluminium hydride was accomplished in a similar way to that of the methyl ester. Approximately 400 mg of 26,27-dinor-25-isopropyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-p-toluene sulphonate was reduced with lithium aluminium hydride (40 mg) in tetrahydrofuran. The reaction product was isolated from the chloroform extract by column chromatography on Kieselgel 60 (12 g) prepared in dichloromethane. Sequential elution with dichloromethane and 5% ethyl acetate:95% chloroform separated the protected  $C_{28}$  (Fig. 3.12) which crystallised from acetone to give white crystals (160 mg). The yield of product is variable and gives 50-60% yield mp 115-119°C.



IR  $\nu$ C-H at 1420  $\nu$ C-H 2840, 2440, 2620, 2710.

at 1350

$M^+$  no molecular ion was detected.

M- Dihydropyran (400) and M- dihydropyran and a hydroxyl group (382) were major bands visualised.

26,27-dinor-25-isopropyl-cholest-5-en-3 $\beta$ -ol (C<sub>28</sub>, Fig. 3.13)

26,27-dinor-25-isopropyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-ene (150 mg) was added to ethanol (10 ml) containing 5 drops of concentrated hydrochloric acid. The solution was heated to 50°C when the protected product dissolved leaving a colourless solution. The heating process was continued for 30 minutes then a few drops of water were added and the volume of the ethanol was reduced under nitrogen. Colourless needle-like crystals were deposited during this time, which were further purified by recrystallisation from methanol.

110 mg (88% yield) m.p. 130°C.

IR  $\nu$ C-OH 3400;  $\nu$ C-H 2880.

n.m.r. 5.4 t (C<sub>5</sub>), 4.0 s (OH at 3), 9 m (saturated alkyl).

MS  $M^+$  400.

26,27-dinor-3 $\beta$ -2'-tetrahydropyranyloxycholest-5-en-24-ol

This synthesis is similar to the Grignard reaction described previously. It only differs in the alkyl halide used to prepare the Grignard reagent. 3 $\beta$ -2'-Tetrahydropyranyloxy-5-cholesten-24-ol (620 mg) was added in dry benzene (15 ml) to methyl magnesium iodide prepared from freshly distilled iodomethane (2 ml) and magnesium (240 mg). The Grignard product was isolated and subjected to column chromatography on silica gel, whence it was isolated by elution with dichloromethane followed by 5% ethyl acetate:95% chloroform.



0.5 g (78% yield).

IR  $\nu$ C-OH 3400;  $\nu$ C-H 2840, 2440, 2620, 2710.

n.m.r. 0.9 t (saturated alkyl), 3.55 g (OH at C<sub>24</sub>), 5.33 d (C<sub>5</sub>).

26,27-Dinor-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-ene

The hydroxyl group of 26,27-dinor-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-ol (500 mg) was activated by the formation of a p-toluene sulphonate ester by reaction with an equal amount of p-toluene-sulphonyl chloride in pyridine (2 ml). The method used is identical to that for the methyl ester and the extracted steroid is concentrated and dried. Following the synthetic scheme for production of 3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-ol, the p-toluene sulphonate ester grouping is eliminated by reduction with lithium aluminium hydride (100 mg) and the compound is isolated by column chromatography on Kieselgel 60. Elution with 5% ethyl acetate:95% chloroform gives a material which crystallises from acetone (220 mg) yield of 50%.

m.p. 104°C.

IR  $\nu$ C-H, 2840, 2710, 2620, 2440.

n.m.r. 5.35  $\delta$  (C at 5), 4.7 (H at 2a), 1 (saturated alkyl).

26,27-Dinor-cholest-5-en-3 $\beta$ -ol (C<sub>25</sub>, Fig. 3.18)

The free sterol was liberated by acid hydrolysis as described previously, 200 mg yielding 105 mg (60%) of 26,27-dinor-cholest-5-en-3 $\beta$ -ol which recrystallised from methanol giving a white powder.

m.p. 132-133°C.

IR  $\nu$ C-OH 3400;  $\nu$ C-H 2840.

n.m.r. 3.6 s (OH at 3); 5.4 (C at C<sub>5</sub>); 0.9 m (saturated alkyl).

M.S. M<sup>+</sup> 358.

26,27-Dinor-25-butyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-en-24-ol

The Grignard addition reaction was performed as for the addition of the 1 bromopentane with the following differences: 26,27-dinor-25-butyl-3 $\beta$ -2'-tetrahydropyranyl cholest-5-en-24-ol was prepared by the addition of 3 $\beta$ -2'-tetrahydropyranyloxy-5-cholesten-24-ol (700 mg) to a Grignard complex prepared from freshly distilled 1-iodopentane (2 ml) and roasted magnesium turnings (240 mg). The addition product formed was isolated by column chromatography as previously described, and 500 mg was isolated (60% yield).

IR  $\nu$ C-OH 3400,  $\nu$ C-H 2850, 2440, 2620, 2710.

26,27-Dinor-25-butyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-ene

The foregoing alcohol was converted to 26,27-dinor-25-butyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-ene by an initial protection step followed by lithium aluminium hydride reduction.

The routine tosylation with 450 mg of starting material gave a heat labile product which was difficult to weigh accurately. Infra red analysis showed that it contained an aromatic group ( $\nu$  C=C 1600) and had lost a C-OH grouping. In all other respects it resembled the starting material (pure by thin layer chromatography but with a greater R<sub>f</sub> value). The conversion to 26,27-dinor-25-butyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-ene was accompanied by 50 mg of lithium aluminium hydride. Extraction by chloroform and silica column chromatography produced in the 5% ethyl acetate:95% <sup>chloroform</sup> eluent a material which crystallises from acetone to give white transparent needles. 200 mg (44% yield of product from 26,27-dinor-25-butyl-3 $\beta$ -2'-tetrahydropyranyloxy-cholest-5-ene).

m.p. 116-117°C.

IR  $\nu$ C-H 2860, 2440, 2620, 2710.

n.m.r. 5.35 t (C<sub>5</sub>), 4.7 s (H 2a), 0.96 m (saturated alkyl).

26,27-Dinor-25-butyl-cholest-5-en-3 $\beta$ -ol (C<sub>29</sub>, Fig. 3.19)

The free sterol was prepared by acid hydrolysis of the tetrahydropyranyl ether (180 mg) in a similar manner to that previously described. The resulting needles were crystallised from methanol.

90 mg 64% yield.

m.p. 115-119°C.

IR  $\nu$ C-OH 3400;  $\nu$ C-H 2850.

n.m.r. 5.38 t (C<sub>5</sub>); 9 m (saturated alkyl), 3.5 s (OH at 3).

M.S. M<sup>+</sup> 414.

3 $\beta$ -2'-Tetrahydropyranyloxy-5-cholen-24-p-toluene sulphonate

3 $\beta$ -2'-Tetrahydropyranyloxy-5-cholen-24-ol (300 mg) was converted to the corresponding p-toluene sulphonate by the addition of excess p-toluene sulphonyl chloride (300 mg) in 1.2 ml of dry pyridine.

The method used was identical to that described in the synthesis of 5 $\beta$ -cholan-3 $\alpha$ ,6 $\alpha$ -diol methyl ester di-p-toluene sulphonate.

The product was concentrated from a chloroform extract by evaporation under nitrogen and the residual solvent was removed by vacuum

desiccation. Thin layer chromatography of the sterol mixture showed the presence of a single non-polar material (330 mg) in 80% yield, infra red analysis of which showed that it possessed (a) an aromatic group (1600 cm<sup>-1</sup>) and (b) C-H bonds (2850 cm<sup>-1</sup>, 2440, 2620, 2710).

Due to the reported lability of tosylates (72) it was considered to be of sufficient purity for the subsequent reduction step.

The primary tosyl ester formed in the reaction was reduced by lithium aluminium hydride with the formation of the corresponding saturated alkane. 5 $\alpha$ -Cholen-3 $\beta$ -tetrahydropyranyloxy-24-p-toluene sulphonate (330 mg) were reacted with an excess of lithium aluminium hydride (40 mg) in freshly distilled tetrahydrofuran (20 ml) by refluxing the solution for four hours in dry apparatus containing a

calcium chloride capped condenser. The product was isolated in the previously described manner and the mixture of three products was resolved by column chromatography on silica gel. The column was prepared in dichloromethane and the mixture was resolved by elution with chloroform and 230 mg (99% yield) of white crystals were prepared from acetone.

m.p. 118-120°C.

IR  $\nu$ -C-H 2880, 2440, 2620, 2710.

n.m.r. 5.35 t ( $C_5$ ), 4.7 s (H at 2a), 9 m (saturated alkyl).

Mass spectrometry - no molecular ion is detected. The major peak observed has a molecular weight of 328. This value could be obtained by cleavage at the 3 position with loss of the dihydropyran and the hydroxyl group.

5 $\alpha$ -Cholen-3 $\beta$ -ol ( $C_{24}$ , Fig. 3.23)

The foregoing tetrahydropyranyl ether (230 mg) was hydrolysed by reflux with ethanolic hydrochloric acid as described in the production of  $C_{25}$ ,  $C_{28}$  and  $C_{29}$ . Evaporation of excess solvent under nitrogen and caused the precipitation of needle-like crystals which were recrystallised from methanol. 120 mg (65%).

m.p. 129-132°C.

IR  $\nu$ C-H 2820,  $\nu$ C-OH 3400.

n.m.r. 5.35 t ( $C_5$ ); 4.0 s (H at 2), 9 m (saturated alkyl).

M.S.  $M^+$  344.

## CHAPTER 4

### SPIN LABEL STUDIES OF CHOLESTEROL ANALOGUE:

#### PHOSPHOLIPID INTERACTIONS



#### 4.1 Introduction

##### resonance

Electron paramagnetic (epr) is a technique that has been widely used in determining intermolecular relationships in membranes. One application of this physical method to the study of membrane structure is by incorporating a spin labelled molecule, formed by attaching a stable free radical to a membrane component, into the membrane. Information regarding the spin label's immediate environment can be derived from the first derivative epr spectrum of a spin label thus incorporated. For example, if the spin labelled molecule is rotating rapidly and randomly in its environment, then a sharp 3 line spectrum is obtained whereas if the molecule is rotating more slowly then the spectrum is increasingly broadened due to a restricted motion of the spin label (83).

Epr has been used to define the role of cholesterol in membranes, particularly model membranes, composed of defined proportions of spin label, cholesterol and phospholipid. A quantitative method for determining the effect of the incorporation of a sterol on the nature of the surrounding phospholipid has been employed by Suckling and Boyd (30) following Hubbell and McConnell (84). Using the 3NC spin label they incorporated an increasing proportion of cholesterol into egg yolk phosphatidylcholine liposomes and monitored the change in the epr spectrum. As the proportion of cholesterol increased, the spectral line widths increased with a consequent broadening of the spectrum due to restriction of the spin label's movement in the phospholipid: cholesterol mixture. Thus, by measuring this spectral change they were able to calculate a value for the restriction of the spin label motion in the presence of a defined percentage of cholesterol.



This was termed the order parameter ( $S$ ) and was an index of the rigidity of the molecular environment surrounding a spin label. This increased ordering could be attributed to an extension of the fatty acyl chains and a decreased amplitude of motion of the long axis of the fatty acyl chains (85).

The role of cholesterol in membrane structures has also been investigated in liposomes prepared from saturated phospholipids such as dipalmitoyl phosphatidylcholine (DPPC). Using the cholestane spin label (3NC) Hemminga (86) showed that the incorporation of small amounts of cholesterol (10 mole per cent) altered the orientation of the DPPC fatty acid side chains in relation to the bilayer normal. Furthermore the interaction of sterol with phospholipid has also been studied by monitoring its effect on the sharp phase transition of DPPC. The spin label Tempo (Fig. 2.5) can be used to indicate the phase transition temperature due to its different solubilities in the gel and liquid crystal phases of phospholipids and has been shown to give a wider range of values for the phase transition than differential scanning calorimetry (87). The transition temperature of a pure phospholipid depends both on the chain length and on the head-group of the phospholipid (88). The transition temperatures of phospholipids containing *cis* unsaturated double bonds are much lower than those of the corresponding saturated analogues. Cholesterol acted as a fluidity regulator fluidising lipids below their transition temperature and rigidifying those above this transition temperature (32).

Thus far the observed properties of cholesterol have been attributed mainly to the presence of the rigid steroid nucleus. The monolayer studies of Demel et al. (89) have shown that in

addition a  $3\beta$  hydroxy group and an intact side chain were required for optimal sterol phospholipid interaction, but the exact length of the side chain for optimal sterol phospholipid interaction remained undefined. In order to define the role of the side chain in cholesterol:phospholipid interactions the cholesterol analogues described in Chapter 3 were incorporated into liposomes containing saturated or unsaturated phospholipids and a series of spin labels.

The following sections describe

- (a) experiments to determine the effect of cholesterol analogues on the motion of two steroid spin labels intercalated in egg yolk phosphatidylcholine liposomes and oriented films,
- (b) experiments that monitor the effect of cholesterol on the phase transition of defined lipid species, and
- (c) the effect of amphotericin B and ascorbate on spin label spectra.

#### 4.2 Cholesterol:phospholipid interaction monitored by 3NC spin label

The spin label 3NC was chosen because of its close similarity to cholesterol. The monolayer studies of Cadenhead et al. (90) and Tinoco et al. (91) showed that although 3NC was less polar than cholesterol and did not condense phosphatidylcholine to the same extent as cholesterol in a monolayer of 3NC and phosphatidylcholine, it was a reasonably good analogue for cholesterol.

In liposomes 3NC is thought to orient with its long axis preferentially along the bilayer normal with the nitroxy group near the aqueous interface and it is capable of rapid rotation about its long axis. In addition the long axis performs an angular motion of limited amplitude (92). For this spin label the magnitude of the perpendicular splitting is greater than the

The results of the incorporation of a series of cholesterol side chain analogues on the motion of liposomal bound 25NC and 3NC spin labels are presented in figures 4.1-4.12

$\mu$ mole C <sub>24</sub>	3NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.22
1.0	50n mole	4.0	0.24
1.5	50n mole	3.5	0.28
2.0	50n mole	3.0	0.29
2.5	50n mole	2.5	0.32

Table 4.1

Results in tables 4.1-4.12 are  $\pm$  0.01 and are the means of at least 4 determinations.

$\mu$ mole C <sub>25</sub>	3NC \	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.235
1.0	50n mole	4.0	0.235
1.5	50n mole	3.5	0.285
2.0	50n mole	3.0	0.297
2.5	50n mole	2.5	0.353

Table 4.2

$\mu$ mole $C_{27}$	3NC	$\mu$ mole E.Y.P C.	( S )
0.5	50n mole	4.5	0.24
1.0	50n mole	4.0	0.255
1.5	50n mole	3.5	0.295
2.0	50n mole	3.0	0.34
2.5	50n mole	2.5	0.396

Table 4.3

$\mu$ mole $C_{28}$	3NC	$\mu$ mole E.Y.P.C.	( S )
0.	50n mole	5.0	0.22
0.5	50n mole	4.5	0.233
1.0	50n mole	4.0	0.25
1.5	50n mole	3.5	0.27
2.0	50n mole	3.0	0.305
2.5	50n mole	2.5	0.339

Table 4.4

$\mu$ mole $C_{29}$	3NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.25
1.0	50n mole	4.0	0.27
1.5	50n mole	3.5	0.285
2.0	50n mole	3.0	0.31
2.5	50n mole	2.5	0.347

Table 4.5

$\mu$ mole sitosterol	3 NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.29
1.0	50n mole	4.0	0.31
1.5	50n mole	3.5	0.32
2.0	50n mole	3.0	0.34
2.5	50n mole	2.5	0.37

Table 4.6

$\mu$ mole $C_{24}$	25NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.21
1.0	50n mole	4.0	0.25
1.5	50n mole	3.5	0.26
2.0	50n mole	3.0	0.27
2.5	50n mole	2.5	0.27

Table 4.7

$\mu$ mole $C_{25}$	25NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.207
1.0	50n mole	4.0	0.217
1.5	50n mole	3.5	0.24
2.0	50n mole	3.0	0.25
2.5	50n mole	2.5	0.27

Table 4.8

parallel and thus the order parameter (S) is expressed as  $\frac{A_1 - A_{11}}{25G}$  (Section 2, Fig. 2.6).

The results of the incorporation of an increasing molar percentage of cholesterol analogue on the ordering of the 3NC spin label included in an unsonicated egg yolk phosphatidylcholine liposome are shown in Tables 4.1 to 4.6. The order parameter (S) is an indication of the rigidifying effect that the analogue is exerting on the surrounding phosphatidylcholine molecules and its value increases proportionally to the sterol present. Comparison of the (S) values for egg yolk phosphatidylcholine alone with cholesterol analogue-phosphatidylcholine mixture shows the following

- (a) in the presence of 10 mole per cent analogue there is no significant increase in ordering.
- (b) with 20 mole per cent of all four sterols the order parameter is increased by 15 per cent compared to the value for egg yolk phosphatidylcholine. Only when the molar proportion of sterol is increased beyond 30 mole per cent does the different ordering properties of each become apparent. At 40 and 50 mole per cent sterol cholesterol and sitosterol cause an increase (56% at 40 mole percent sterol and 69% at 50 mole per cent sterol) in ordering greater than that due to  $C_{24}$ ,  $C_{25}$ ,  $C_{28}$  and  $C_{29}$  (33% at 40 mole per cent, 55% at 50 mole per cent). Thus the results indicate that with 40 mole per cent sterol, using a probe that is thought to monitor the region of the bilayer adjacent to the aqueous phase, optimal ordering of the surrounding phospholipid is obtained using a sterol with a  $9\overset{O}{A}$  long side chain, i.e. cholesterol or sitosterol.



$\mu$ mole $C_{27}$	25NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.21
1.0	50n mole	4.0	0.23
1.5	50n mole	3.5	0.25
2.0	50n mole	3.0	0.268
2.5	50n mole	2.5	0.29

Table 4.9

$\mu$ mole $C_{28}$	25NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.193
1.0	50n mole	4.0	0.217
1.5	50n mole	3.5	0.235
2.0	50n mole	3.0	0.255
2.5	50n mole	2.5	0.27

Table 4.10

$\mu$ mole $C_{29}$	25NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.215
1.0	50n mole	4.0	0.24
1.5	50n mole	3.5	0.26
2.0	50n mole	3.0	0.275
2.5	50n mole	2.5	0.295

Table 4.11

$\mu$ môle sitosterol	25NC	$\mu$ mole E.Y.PC	( S )
0.5	50n mole	4.5	0.215
1.0	50n mole	4.0	0.240
1.5	50n mole	3.5	0.260
2.0	50n mole	3.0	0.275
2.5	50n mole	2.5	0.295

Table 4.12

#### 4.3 Ordering of 25NC spin label in liposomes containing the cholesterol side chain analogues

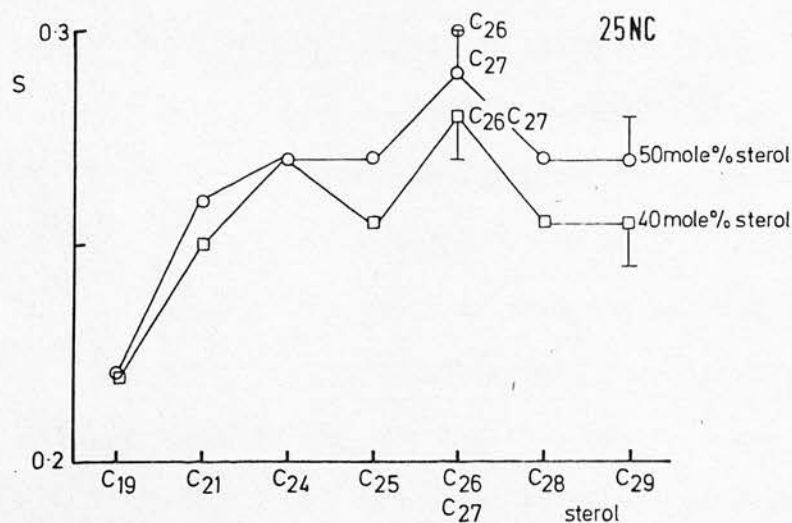
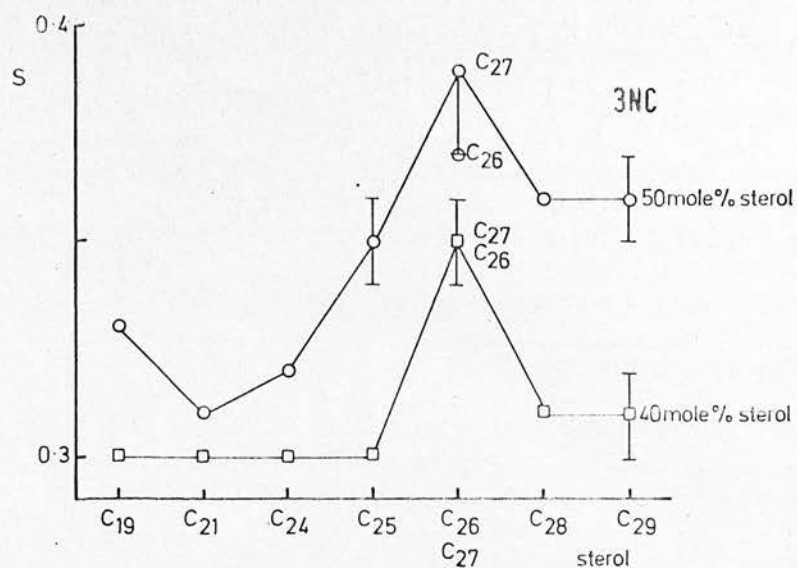
A spin label containing the reporter group in the side chain and thus reporting on a different part of the bilayer from 3NC was incorporated in sterol:egg yolk phosphatidylcholine liposomes.

In contrast to the broad 3NC spectrum (Fig. 2.6a) which is indicative of restricted nitroxyl motion consistent with its role as a reporter of events at the bilayer surface, the 25NC spin label (Fig. 2.6b) spectrum consists of three sharper lines. The sharpness of the spectrum indicates that the nitroxyl group's motion at position 25 is unrestricted by its surrounding environment. In fact the  $C_{25}$  nitroxyl behaviour is more akin to that of a fatty acid spin label and this is reflected in the order parameter calculation. This is consistent with a centre of the bilayer location for the nitroxyl group at carbon 25.

The multilayer liposomal preparation described in the Methods section was employed and the data are included in Tables 4.7-4.12. The previously described increase in ordering with increasing concentration of sterol is once more observed. However there are some differences from the 3NC results.

(a) even with 10 mole per cent sterol there is an increase in spin label ordering varying from 8.9% with  $C_{24}$ ,  $C_{25}$ ,  $C_{28}$  and  $C_{29}$  to 16% with cholesterol and sitosterol.

(b) with 40 mole per cent sterol  $C_{27}$  and sitosterol the order parameter increases by 40% whereas  $C_{24}$ ,  $C_{25}$ ,  $C_{28}$  and  $C_{29}$  only cause a 32% increase.



Comparison of the effect of alteration of the cholesterol side chain on the motion of the 3NC and 25NC spin labels.

Fig. 4.1

(c) in the presence of 50 mole per cent sterol  $C_{27}$  and sitosterol the order parameter increases by 55% whereas  $C_{24}$ ,  $C_{25}$ ,  $C_{28}$  and  $C_{29}$  at the same concentration increase the ordering by 38%.

The 25NC results are slightly different from 3NC but confirm the previously observed trend. If the 40 and 50 mole per cent results for 3NC and 25NC for experiments using  $C_{19}$ ,  $C_{21}$  and  $C_{26}$  are included in a graph of order parameter plotted against analogue carbon number (Fig. 4.1) it is clearly seen that cholesterol causes the maximum increase in order parameter at both sterol concentrations.

#### 4.4 Cholesterol-phospholipid interactions in oriented films

The interaction between cholesterol and phospholipids has been studied in oriented films by Smith et al. (92) and Seelig (93), among others. This system has advantages over a phospholipid dispersion due to the greater resolution of the spectra obtained. In addition the various contributions to the resulting spectra could be more easily identified in this defined system allowing a more detailed analysis of cholesterol interaction than was possible using a liposomal system. Using this method Lapper et al. (94) have defined the order parameter more precisely as the extent to which the long axis of the lipids are oriented preferentially in the direction perpendicular to the lamellar phase. In a further study (95) using oriented films composed of brain lipids and cholesterol-like molecules they have shown that the extent of ordering caused by each sterol depended on the molar proportion of sterol present and the actual structure of each sterol. At 20 mole per cent sterol, cholesterol and ergosterol were equally effective whereas at 5-10 mole per cent ergosterol caused the maximum amount of ordering but its effect decreased beyond 10 mole

per cent. Their conclusions were that for optimal ordering of a brain lipid film, a sterol molecule required a 3 $\beta$  hydroxy group and a non-polar side chain of undefined length (the presence of a hydroxyl group in the side chain prevented the ordering of the surrounding lipid). As cholesterol and cholestanol were equally effective at ordering the surrounding phospholipid molecules, a double bond at C<sub>5</sub> was not thought to be of paramount importance.

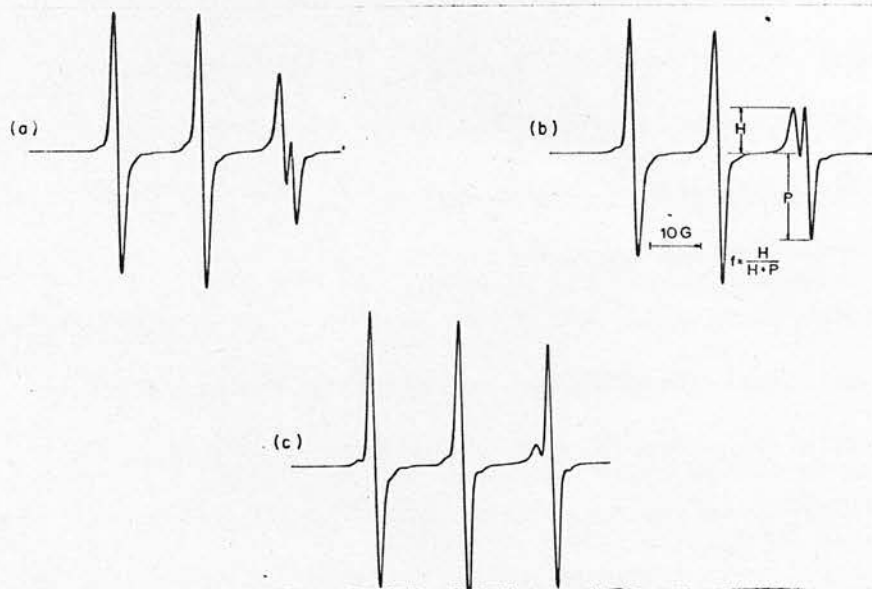
In order to further define the sterol side chain requirements for optimal sterol phospholipid interactions in this system, oriented multi-bilayers of sterol and egg yolk phosphatidylcholine (30:70 mole per cent) containing 1 mole per cent of the 3NC spin label were prepared by Dr. Keith Suckling from hydrated films of the lipids as described by Seeling (93). The spin label spectra were recorded with the film oriented parallel and perpendicular to the applied magnetic field and the results detailed in Table 4.13 were obtained.

This shows that cholesterol caused the maximum increase in order parameter and increasing the side chain beyond 9 $\text{\AA}$  (C<sub>28</sub> and C<sub>29</sub>) resulted in a significant decrease in its value. This absolute value of C<sub>28</sub> and C<sub>29</sub> were intermediate between C<sub>19</sub>, C<sub>21</sub>, C<sub>24</sub>, C<sub>25</sub> and C<sub>27</sub>. This was slightly different from the results obtained with the liposomes where the order parameters were smaller due to averaging of the signals and the values of (S) were similar for C<sub>24</sub>, C<sub>25</sub>, C<sub>28</sub> and C<sub>29</sub>.

#### 4.5 The effect of the cholesterol analogues on dipalmitoyl phosphatidylcholine (DPPC) monitored by 2,2,6,6-tetramethylpiperidine-I-oxyl (Tempo)

The experiments in Section 5 were designed to show that the





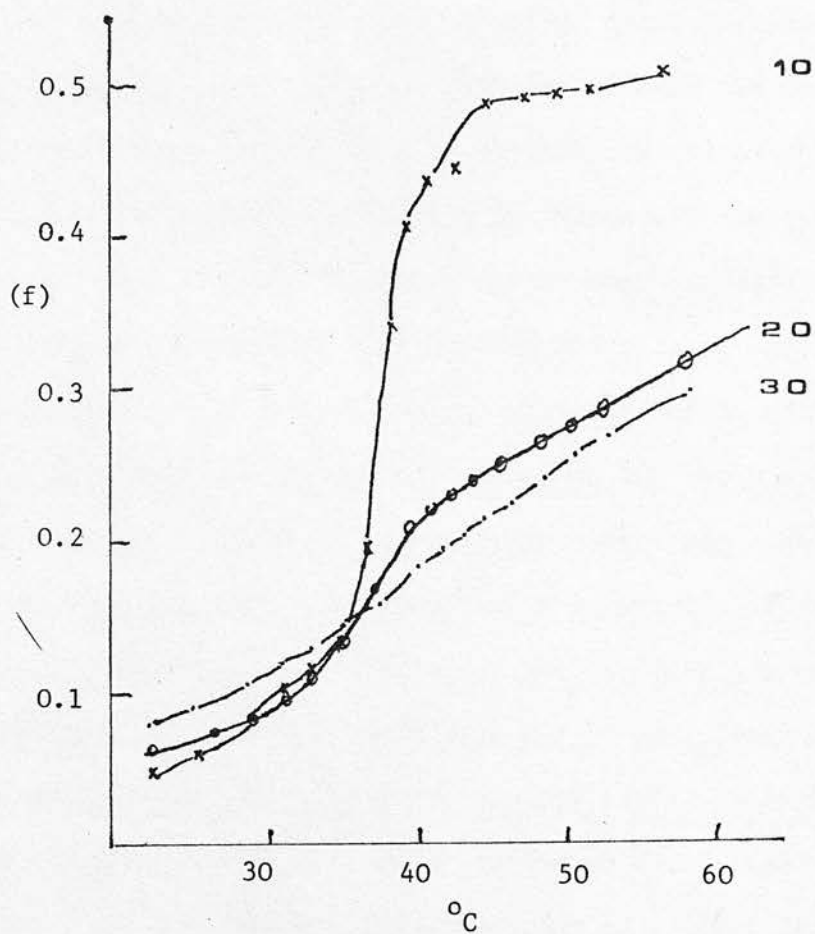
The epr spectrum of Tempo in an aqueous dispersion of the phospholipid, dipalmitoyl-phosphatidylcholine (a) above the phase transition temperature ( $57^{\circ}\text{C}$ ) (b) nearly equal to the transition temperature ( $37^{\circ}\text{C}$ ) (c) below the transition temp. ( $22^{\circ}\text{C}$ ). Parameter (f) is an approximation to the fraction of Tempo dissolved in the hydrocarbon phase and is measured from the splitting of the high field line.

Fig. 4.2

pattern of interaction observed with the egg yolk phosphatidylcholine could be repeated when a dynamic event, namely the phase transition of a saturated phospholipid, was studied by a different spin label.

The basic design of these experiments concerned monitoring the change in solubility of Tempo in the lipid phase when a liposome containing the saturated phospholipid, dipalmitoyl phosphatidylcholine, in the presence of a cholesterol side chain analogue, was warmed from 22°C to 57°C. Fig. 4.2 shows the first derivative epr spectra of a  $10^{-3}$  M concentration of Tempo in an aqueous dispersion of dipalmitoyl phosphatidylcholine at 3 temperatures. The concentration of Tempo is kept low in order to minimise interaction between spin labels which results in a broadening of the spectra. Each spectrum is a superposition of 2 spectra - one due to Tempo dissolved in the fluid lipid hydrophobic phase and the other due to Tempo in the aqueous phase.

Small differences in the nitroxide isotropic hyperfine coupling and the 'g' factors in each environment result in a partial resolution of the high field line (X band) whereas the low and middle lines are not resolved. The spectral parameter (f) was calculated according to the procedure of Shimshick & McConnell (69). At room temperature dipalmitoyl phosphatidylcholine is in a gel phase in which the hydrocarbon chains are tightly packed in a hexagonal lattice (96). The tight packing is reflected in the isotropic 22°C spectrum of Tempo (Fig. 4.2). As the temperature is increased to 30-35°C, which is below the phase transition temperature of DPPC (41°C) there is a slight increase in the Tempo solubility. This is thought to be due to the formation of a disordered liquid crystalline lipid region



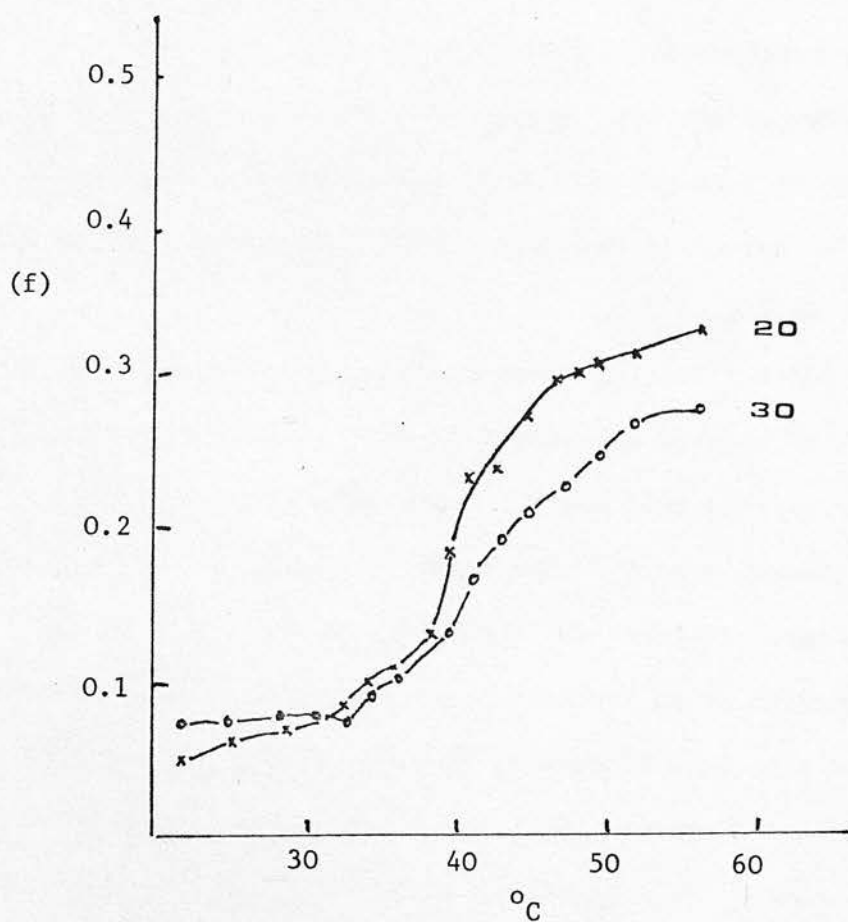
Tempo partitioning into DPPC liposomes  
containing 10, 20 or 30 mole %  $C_{19}$ .

Fig. 4.3

within the gel matrix. The partial disruption of the hexagonal matrix is thought to be occurring in one of three ways

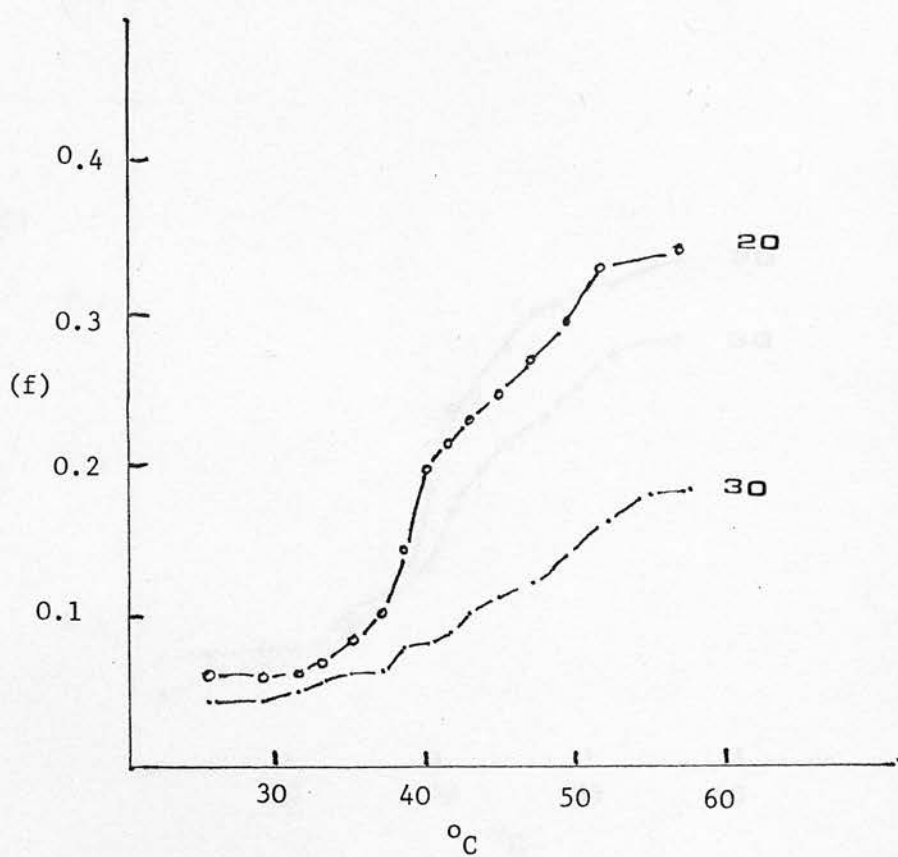
- (1) as a result of changes on the configuration of the hydrocarbon chains from all trans to a trans-gauche mixture (33).
- (2) due to a change in the orientation of the lipid fatty acid chains from being tilted with respect to the bilayer plane to being oriented perpendicular to the bilayer plane (97) thus allowing rotation about their long axes.
- (3) Levine et al. (98) suggest that the head group conformations change at this temperature from being parallel to the bilayer surface to being perpendicular to it. The pretransition spectrum is shown in Fig. 4.2.

On further heating there is an increase in the cooperative interaction between adjacent hydrocarbon chains resulting in sharp transition from the gel phase to a completely fluid crystal phase. At 57°C the solubility of Tempo is maximal (Fig. 4.3a). Figures 4.3 and 4.10 show the effect of the incorporation of an increasing molar proportion of cholesterol analogue into DPPC liposomes, on Tempo partition. When the ratio of DPPC to analogue was 10:1, of the sterols tested only C<sub>26</sub> and C<sub>27</sub> caused any significant deviation from the melting profile of the pure phospholipid (4.11). As the percentage of sterol was increased to 20%, the value of (f) decreased due to increased membrane rigidity and differences in the analogues relative abilities to exclude the spin label became apparent (Fig. 4.12). The analogues fell into two main groups with C<sub>19</sub>, C<sub>21</sub>, C<sub>24</sub> and C<sub>25</sub> causing a 47% reduction in Tempo solubility (relative to DPPC) at 47°C as compared to a 56% reduction with C<sub>26</sub>, C<sub>27</sub> and C<sub>28</sub>.



Tempo partitioning into DPPC liposomes  
containing 20 or 30 mole % C<sub>21</sub>.

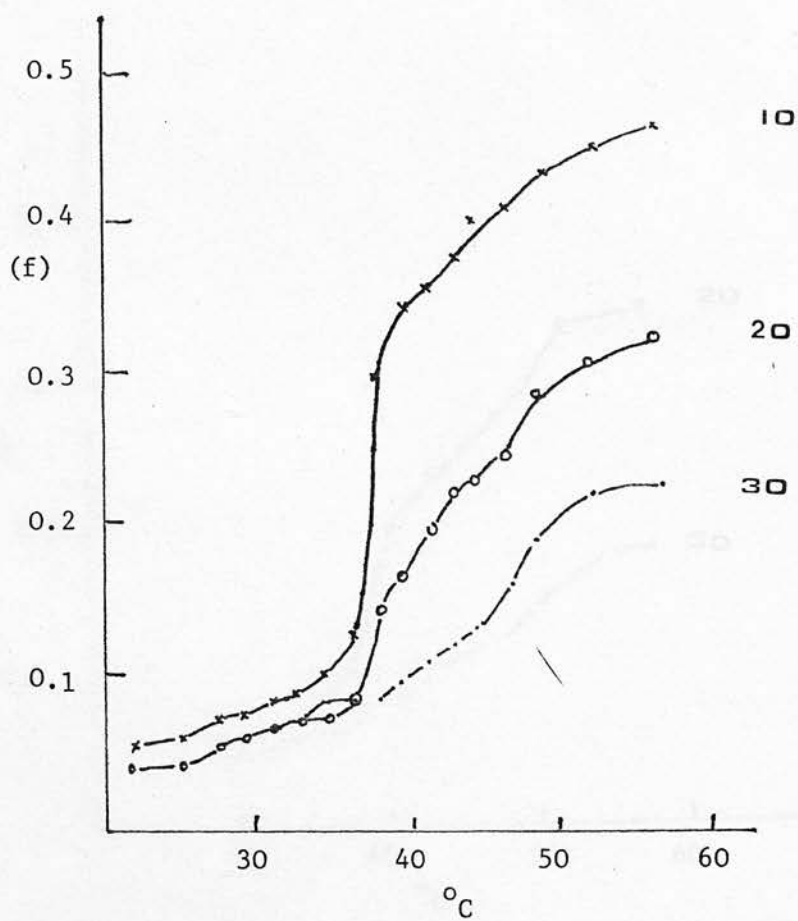
Fig. 4.4



Tempo partition into DPPC liposomes  
containing 20 or 30 mole % C<sub>24</sub>.

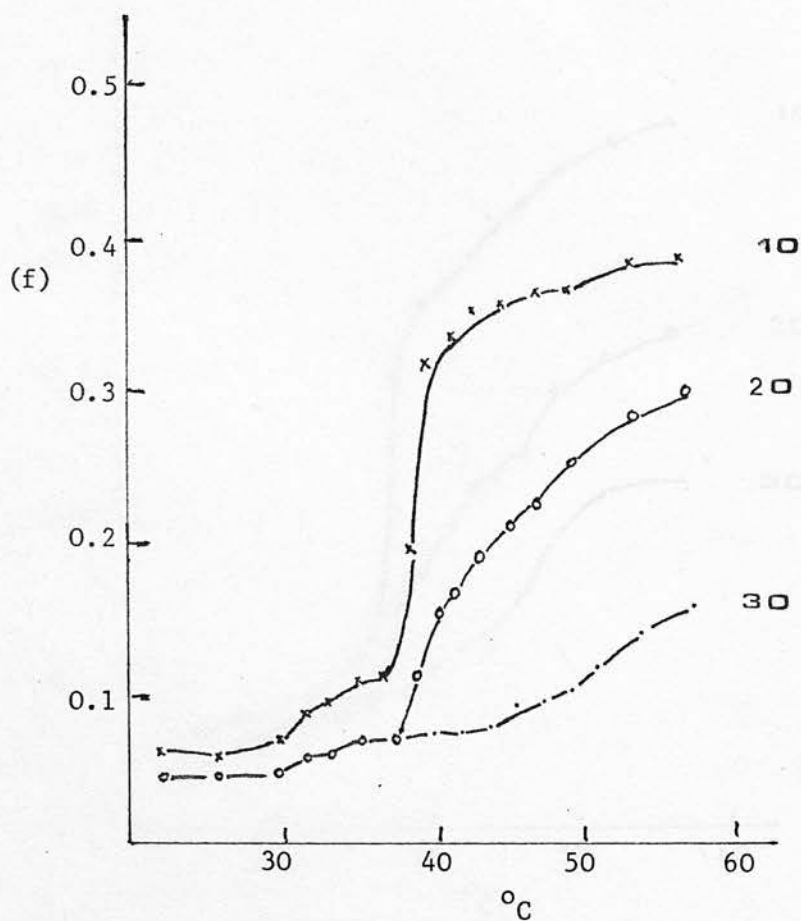
Fig. 4.5





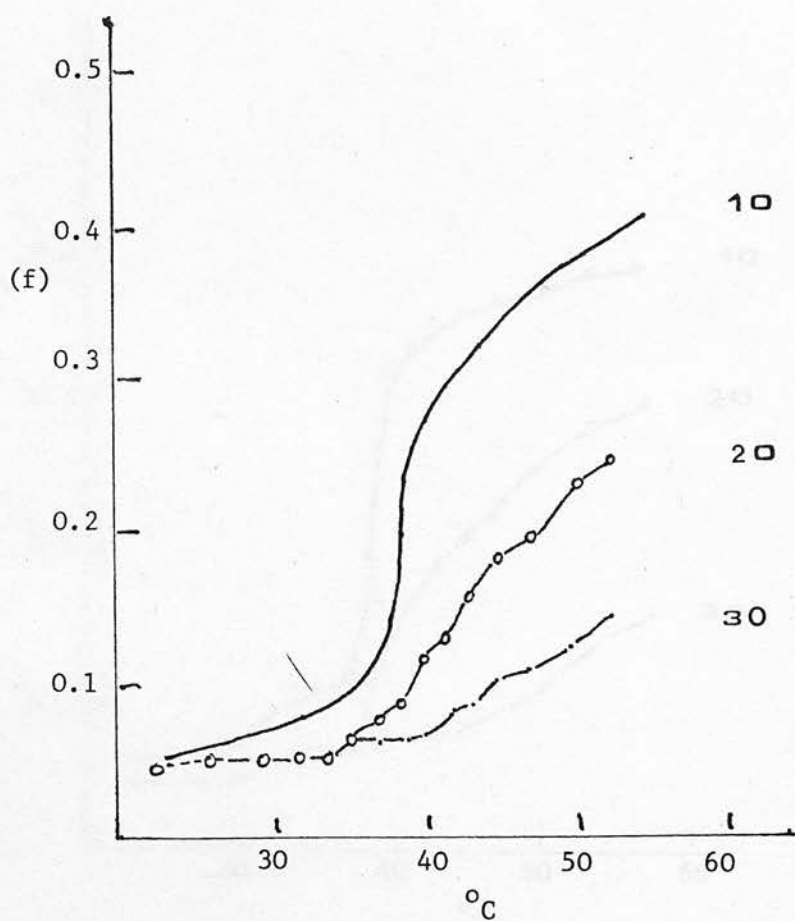
Tempo partitioning into DPPC liposomes  
containing 10, 20 or 30 mole % C<sub>25</sub>.

Fig. 4.6



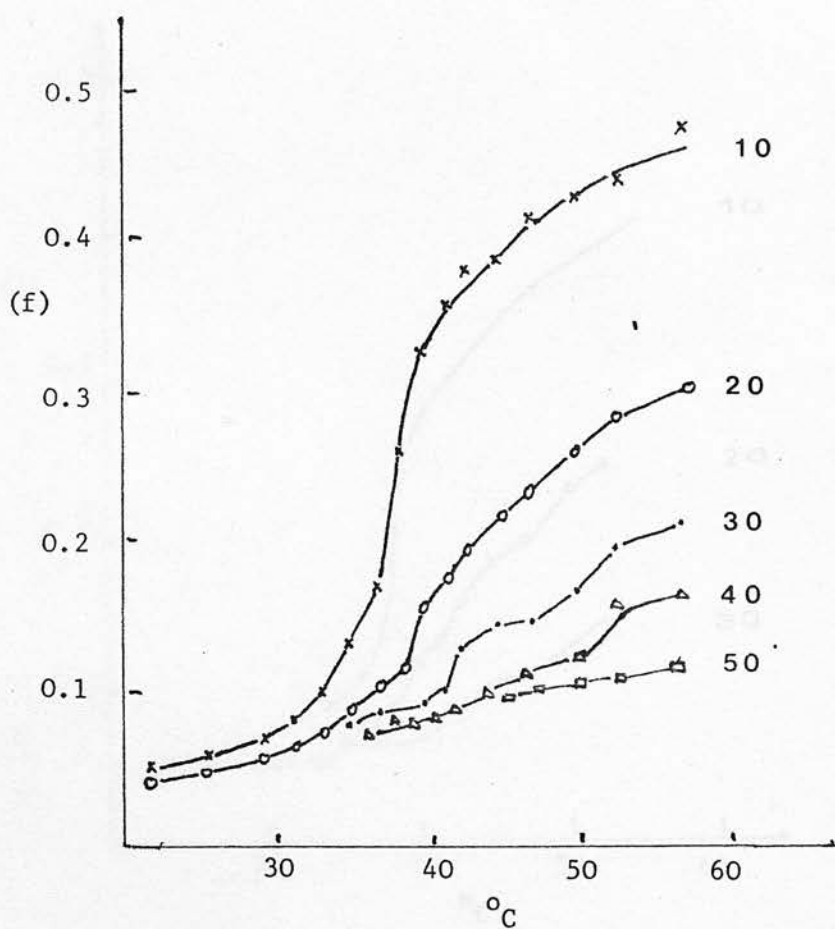
Tempo partitioning into DPPC liposomes  
containing 10, 20 or 30 mole % C<sub>26</sub>.

Fig. 4.7



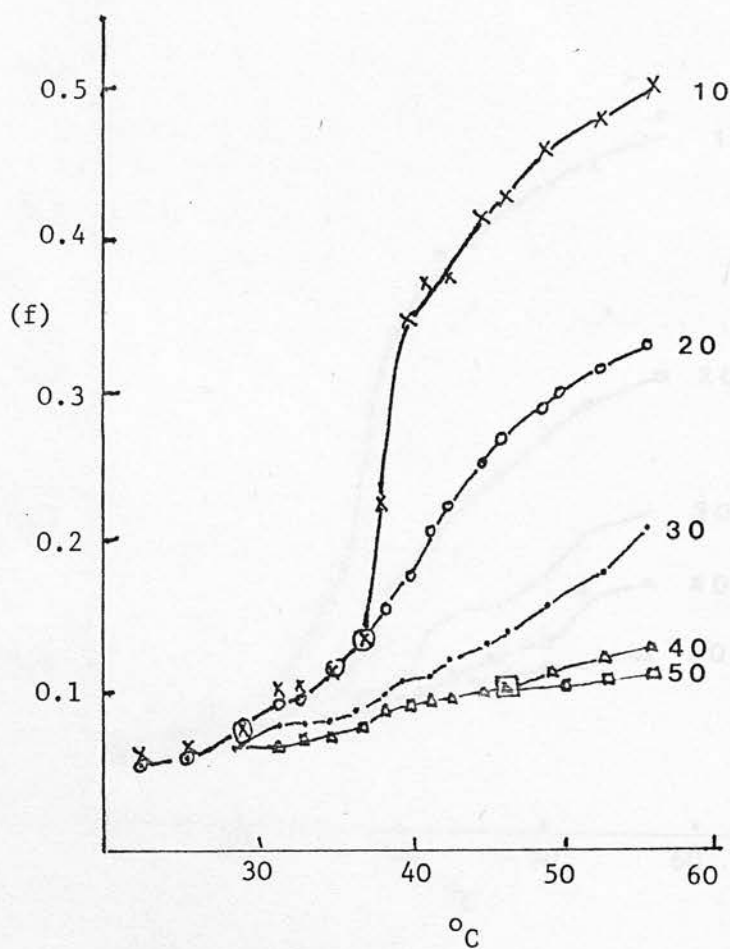
Tempo partitioning into DPPC liposome  
containing 10, 20 or 30 mole % C<sub>27</sub>.

Fig. 4.8



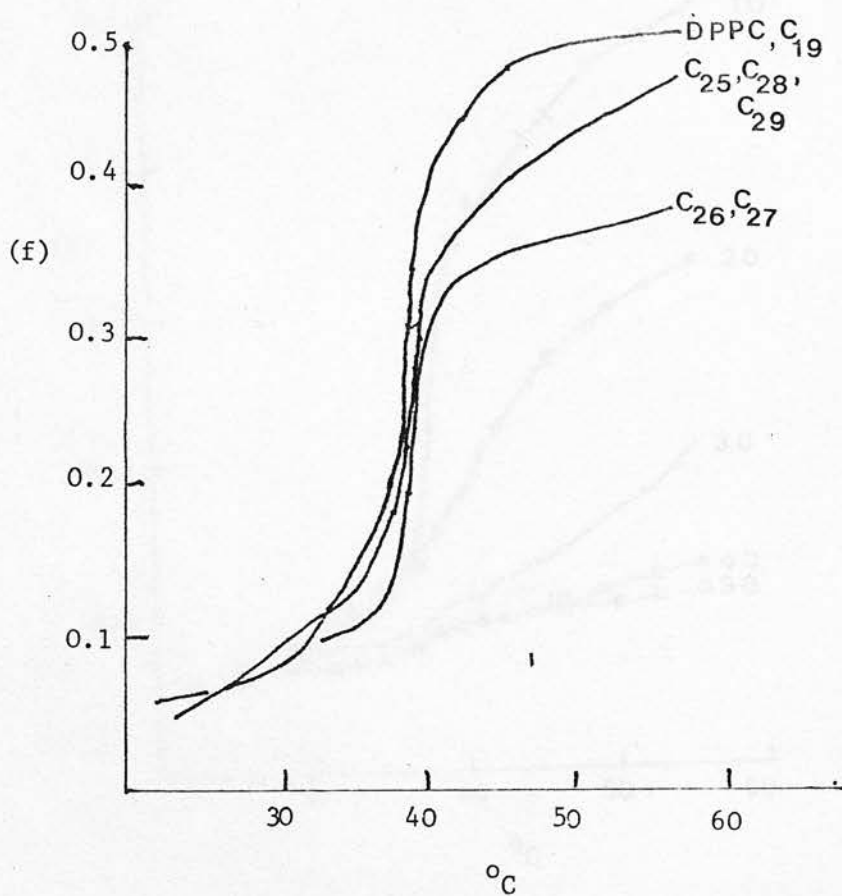
Tempo partitioning into DPPC liposomes  
containing 10, 20, 30, 40 or 50  
mole % C<sub>28</sub>.

Fig. 4.9



Tempo partitioning into DPPC liposomes  
containing 10, 20, 30, 40 or 50  
mole %  $\text{C}_{29}$ .

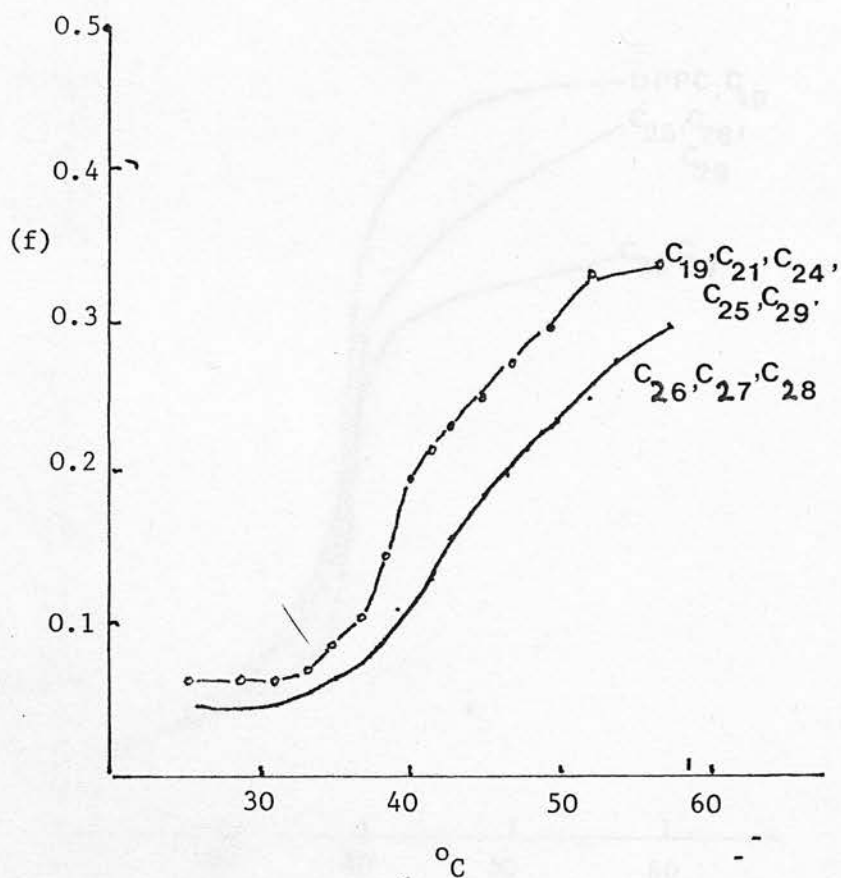
Fig. 4.10



Tempo partitioning into DPPC:  
sterol liposomes containing 10 mole %  
sterol. DPPC alone is similar to C<sub>19</sub>  
results.

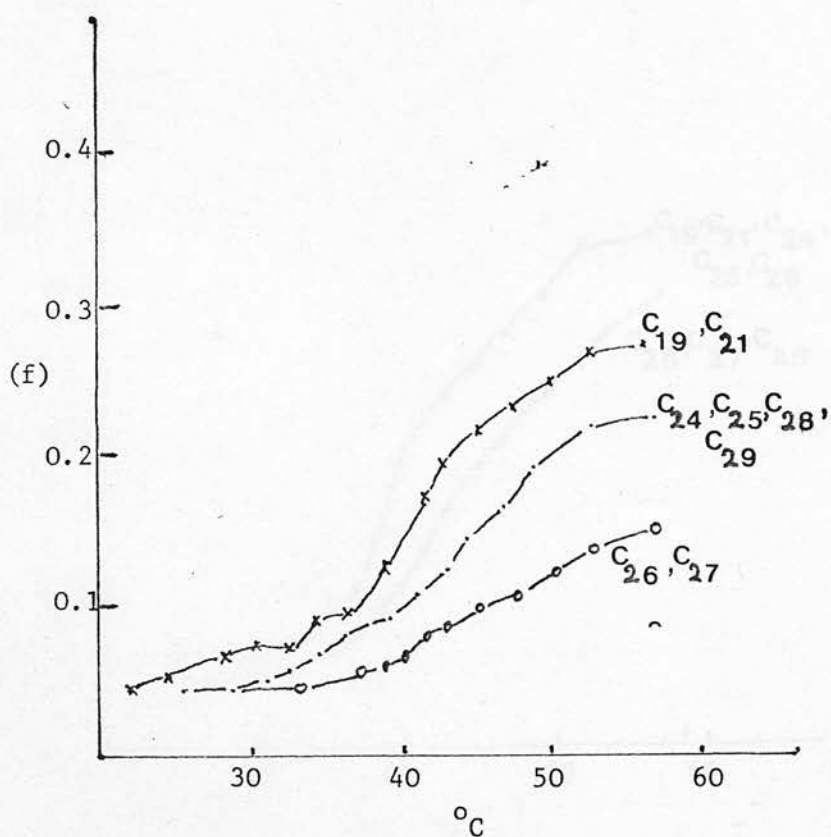
Fig. 4.11





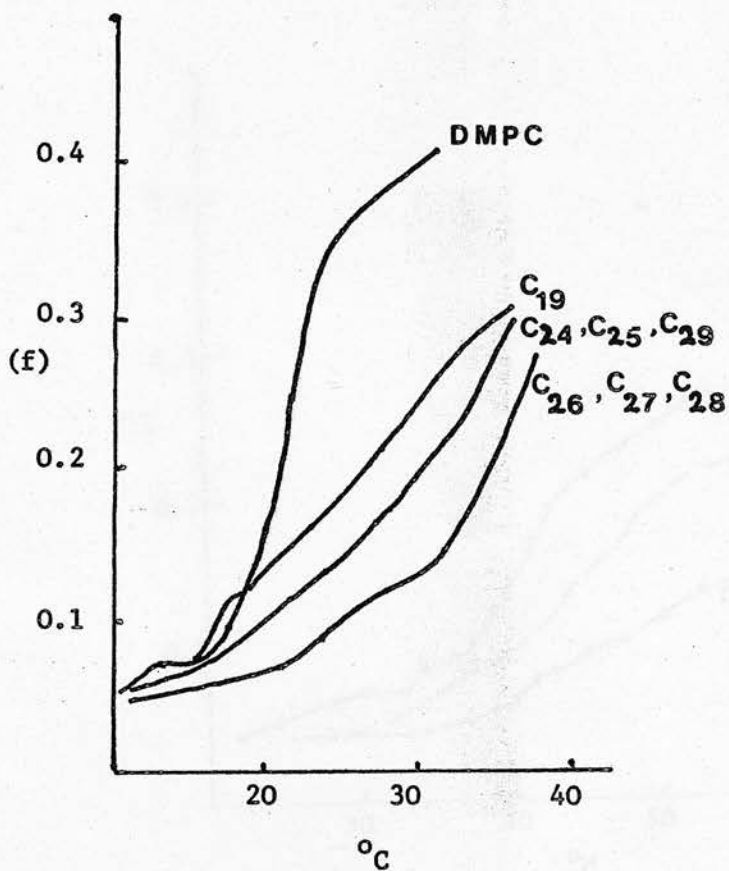
Comparison of the ability of Tempo to partition into DPPC liposomes containing 20 mole % cholesterol analogue.

Fig. 4.12



Comparison of the ability of Tempo to partition into DPPC liposomes containing 30 mole % cholesterol analogue.

Fig. 4.13



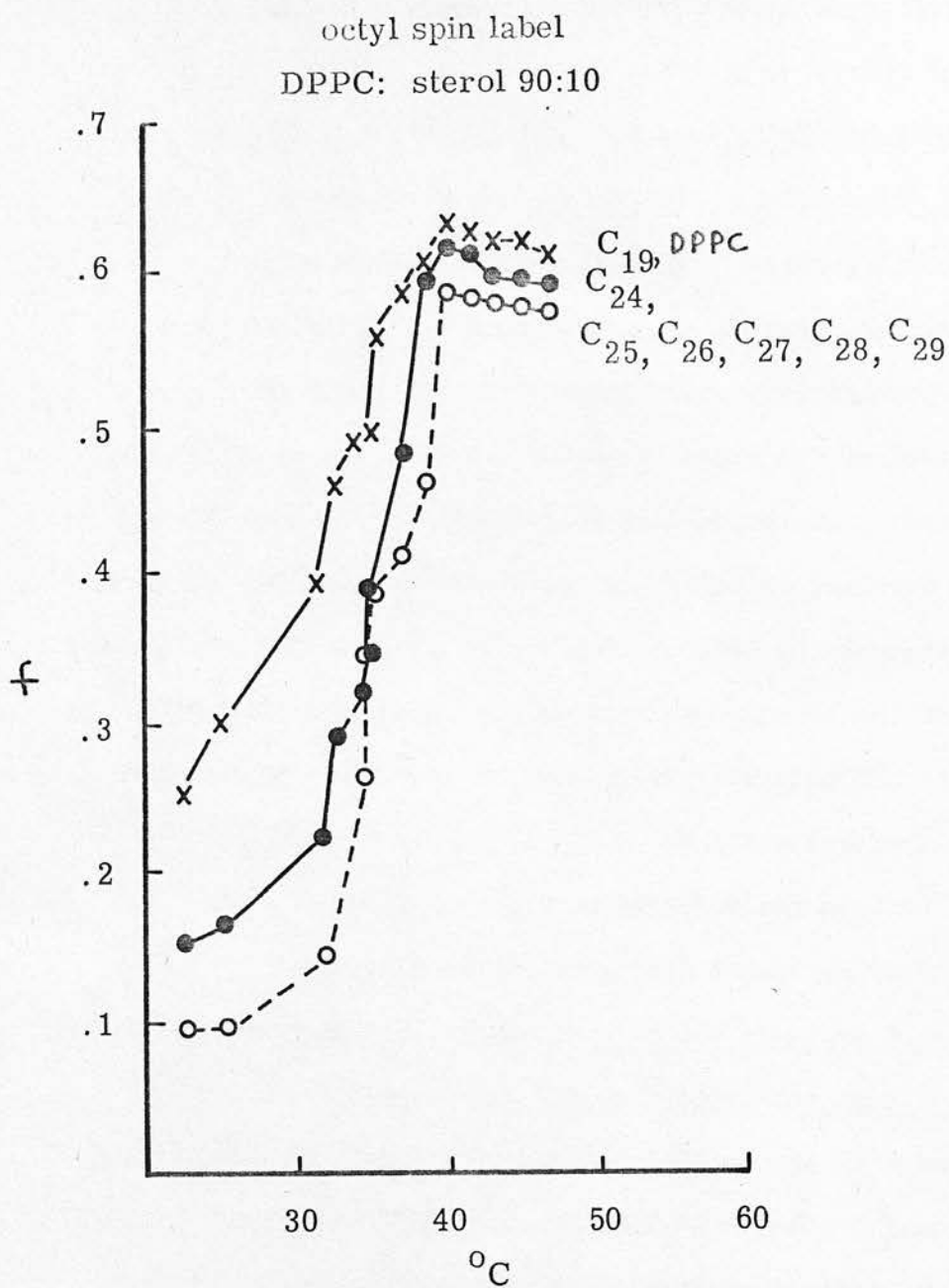
The effect of replacing DPPC by DMPC  
on the partitioning of Tempo into  
liposomes containing 20 mole %  
cholesterol analogue.

Fig. 4.14

On the addition of a further 10 mole per cent sterol a slightly different distribution was obtained -  $C_{19}$  and  $C_{21}$  reduced the (f) value by 50%:  $C_{24}$ ,  $C_{25}$ ,  $C_{28}$  and  $C_{29}$  caused a 70% reduction with  $C_{26}$  and  $C_{27}$  giving a 77% decrease in solubility (Fig. 4.13). At 30 mole per cent sterol the phase transition disappeared. A similar value was calculated from the differential scanning calorimetry experiments of Hin & Sturtevant (99) with cholesterol and DPPC. This disappearance of the phase transition of pure phospholipid in the presence of cholesterol is well documented. Although the mechanism involved is not fully understood, a possible explanation could be that as the molar proportion of cholesterol is increased the size of the cooperative unit of the phospholipid molecules involved in the phase transition, which determines its sharpness (101), will decrease due to intercalation of the cholesterol molecules between the hydrocarbon chains resulting in a cholesterol-phospholipid mixture of intermediate fluidity (32).

#### 4.6 Solubility of Tempo in dimyristoyl phosphatidylcholine containing 20 mole per cent cholesterol side chain analogue

In order to ensure that the side chain pattern established with dipalmitoyl phosphatidylcholine did not depend on a specific phospholipid with 16 carbons in the fatty acid chains, the same range of analogues were incorporated at 20 mole per cent in dimyristoyl phosphatidylcholine (DMPC, 14 carbons in the fatty acyl chains) liposomes. Figure 4.14 shows the results obtained. A similar pattern to that noted for DPPC was observed -  $C_{19}$ ,  $C_{24}$ ,  $C_{25}$ ,  $C_{29}$  and  $C_{26}$ ,  $C_{27}$ ,  $C_{28}$  reducing Tempo solubility by 33, 56 and 67 % respectively at 29°C compared to the results with dimyristoyl phosphatidylcholine. It should be noted that the



The partitioning of 3N8 into DPPC liposomes containing 10 mole % cholesterol analogues.

Fig. 4.15

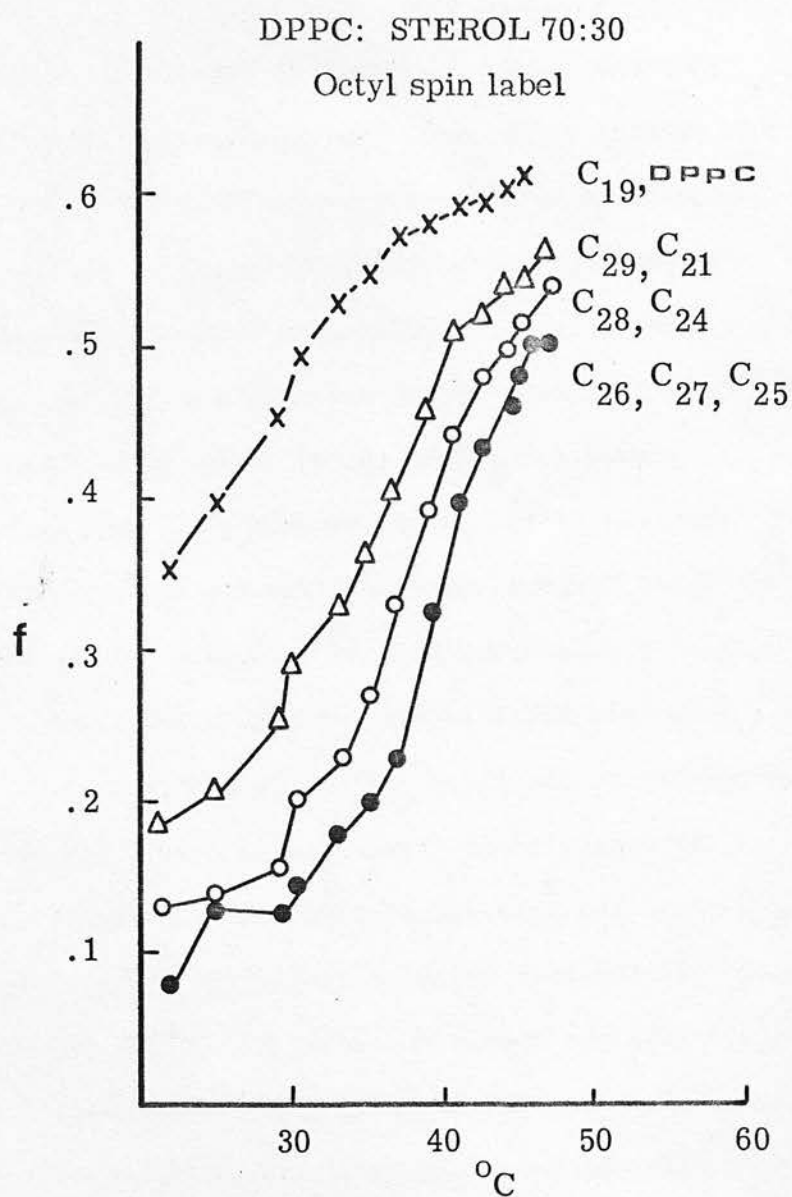
solubility of Tempo was greater in DPPC than DMPC when both phospholipids were in the fluid liquid crystalline phase.

4.7 The effect of cholesterol side chain analogues on the phase transition of dipalmitoyl phosphatidylcholine as monitored by a 3 nitroxide octane (3N8) spin label

The experiments described in Section 4.6 were repeated using another spin label, 3-nitroxy octane, instead of Tempo. The mechanisms by which each spin label indicates the nature of its surroundings are slightly different. As stated previously Tempo is capable of dissolving in both the hydrophobic and hydrophilic regions of the membrane and analysis of the plot of the (f) versus ( $^{\circ}\text{C}$ ) (Fig. 4.14) shows that it defines the lower and upper end of the phase transitions. As the lipid layer rigidifies, Tempo is extruded from the membrane phase, and the lower end of the transition is defined. Using 5N10, an analogous spin label to 3N8, Linden et al. (102) characterised its ability to partition into a lipid bilayer.

They suggested that the aqueous solubility of this nitroxide was limited and measured its lipid solubility by calculating either (a) the peak height ratio of the midfield and high field lines or (b) the apolar and polar high field line heights (Fig. 2.7). In addition this hydrocarbon probe remained in the membrane in the gel phase and appeared to separate laterally in the hydrocarbon phase into the solid or liquid phase. They state that the partitioning abilities prevented 5N10 from detecting the upper limits of the phase transition and only a large reduction in the concentration of liquid hydrocarbon chains resulted in the determination of the lower limit.





The partitioning of 3N8 into DPPC liposomes containing 30 mole % cholesterol side chain analogue.

Fig. 4.16

The results of the 3N8 experiments are illustrated in Figures 4.15 and 4.16. The curves obtained do not resemble those obtained with Tempo and unlike those of Linden et al. (102) do not give a clear indication of the lower end of the phase transition or the actual phase transition temperature. The calculated values for the fluidity parameter are higher than for Tempo in the liquid crystalline phase and the analogues tested only seem to cause a small reduction in the fluidity parameter above the transition temperature.

At temperatures less than  $41^{\circ}\text{C}$  the analogues are seen to exert a greater effect on the solubility of the spin label. Comparison of the (f) values at  $38.5^{\circ}\text{C}$  obtained for DPPC with the analogues DPPC mixture showed that at 10 mole per cent (Fig. 4.15)  $\text{C}_{19}$  had no effect,  $\text{C}_{24}$  caused a 5% reduction whereas  $\text{C}_{25}$ ,  $\text{C}_{26}$ ,  $\text{C}_{27}$ ,  $\text{C}_{28}$  and  $\text{C}_{29}$  reduced the partitioning by 17%. Increasing the proportion of sterol present to 30 mole per cent (Fig. 4.16) still had no effect with  $\text{C}_{19}$  but a 17% reduction was noted with  $\text{C}_{24}$  and  $\text{C}_{28}$  and a 45% reduction with  $\text{C}_{25}$ ,  $\text{C}_{26}$  and  $\text{C}_{27}$ .

#### 4.8 Location of the available spin labels in egg yolk phosphatidyl-choline liposomes

In order to confirm the proposed locations for the spin labels used in this study, Tempo, 3NC, 25NC, 1 nitroxy stearamide (probe for the bilayer surface) and 3N8 (Fig. 2.5) were incorporated into a liposomal structure and solutions of either nickel chloride or ascorbic acid were added. Both solutions removed the spin label signal. Nickel reversibly removed the spin label whereas ascorbate converted the nitroxyl group to hydroxylamine destroying the free radical. Assuming that interaction

between the spin label and the nickel or ascorbate was very rapid, the relative rates of removal of each spin label's signal should give an indication of the location of the spin label's nitroxyl grouping in the liposome membrane.

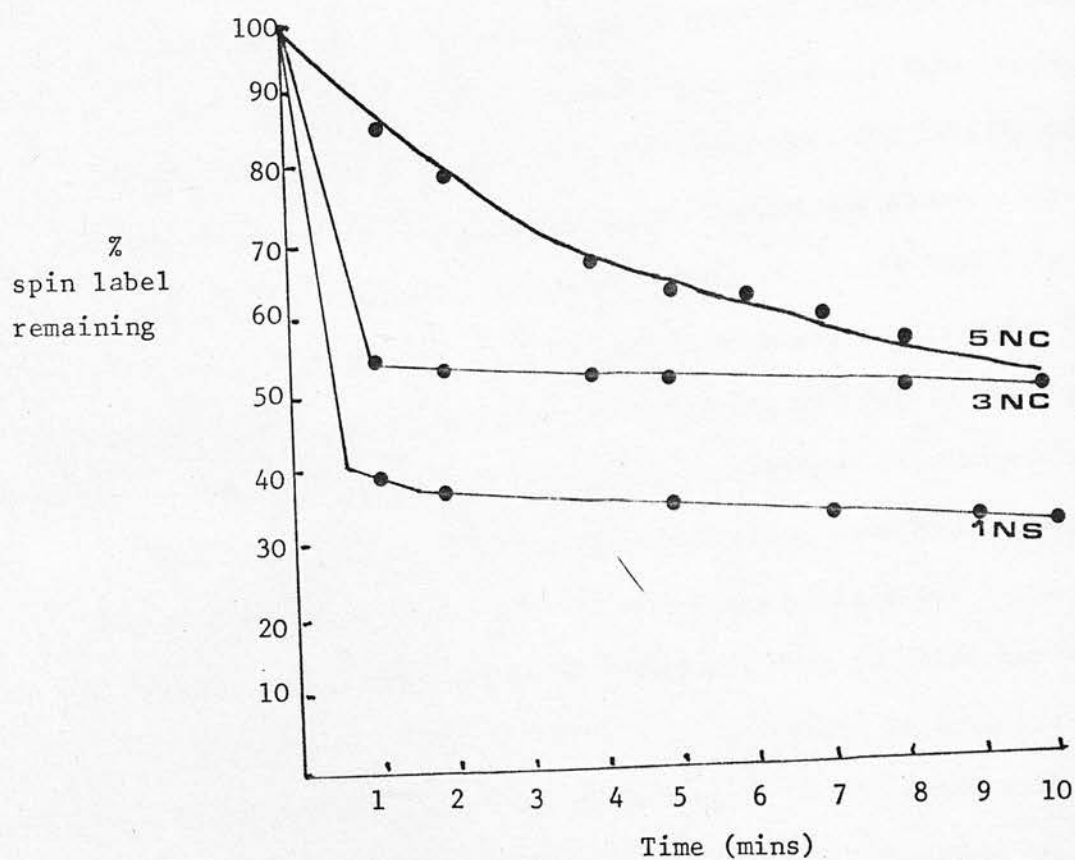
#### Nickel Chloride ( $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ )

In the presence of 100 nmoles of nickel chloride the magnitude of the high field line of an aqueous nitroxide spectrum of Tempo and 3N8 (10 nmoles) was reduced by an average of 44% after five minutes at room temperature. The experiment was repeated with the same spin labels (10 nmoles) incorporated into dipalmitoyl phosphatidylcholine. Equimolar nickel chloride did not reduce the signal obtained but Table 4.14 shows the effect of a 10 fold excess of nickel solution. The high field line of the epr spectrum for each sample was recorded on either side of the phase transition prior to the addition of the nickel when the process was repeated. In the case of Tempo, the aqueous signal seemed most susceptible to the addition of nickel with a resultant increase in the value of (f). 3N8 underwent a 30 per cent reduction in both the hydrophilic and hydrophobic signals in the presence of nickel.

The phase of the lipid did not seem to determine nitroxyl accessibility to the diamagnetic species, but the differences in the spin label's aqueous signal reduction indicated that Tempo is more accessible than the 3N8.

#### Ascorbate

Essentially the method followed was that described by Schreier & Mu<sup>c</sup>cillo et al. (103) with the differences that multilayered liposomes (4 mg egg PC/30 ml buffer) were used and the ascorbate concentration was 1 M not  $10^{-2}$  M. The reduction in



The effect of ascorbate (M) on the signals of spin labels incorporated into egg yolk phosphatidylcholine liposomes.

(For spin label structures see Fig. 2.5)

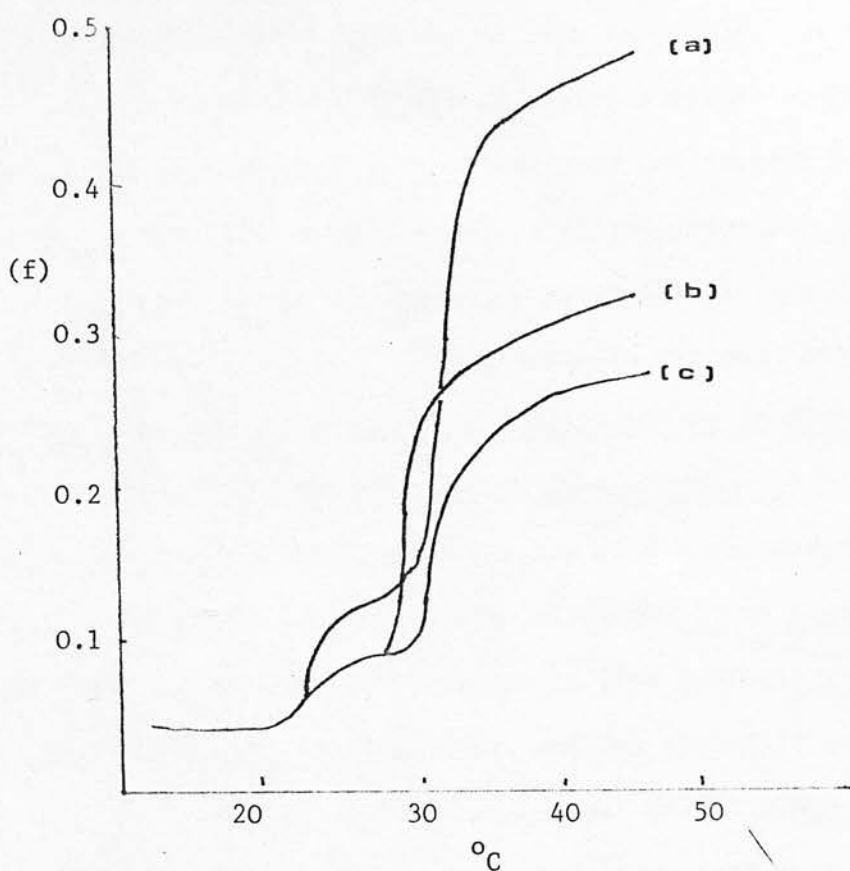
Fig. 4.17

the midline was monitored on the addition of 2 ml of ascorbate to 50 nmoles of the liposomal spin label over a ten minute period. With this concentration of ascorbate each spin label's signal was removed within 30 seconds in aqueous solution.

Figure 4.17 shows the results obtained with the surface probe 1 nitroxystearamide (INS), 3NC, and 25NC. The data seemed to clearly support the view that the 3NC and 25NC nitroxyls are situated at different depths in the bilayer. In addition it appears that in all cases a portion of the spin label is protected from the ascorbate.

#### 4.9 The effect of amphotericin B on Tempo solubility in liposomes prepared from dipalmitoyl phosphatidylcholine

Amphotericin B is one of the polyene antibiotics which comprise a group of cyclic amphipathic lactones, which contain a number (usually 4-7) of conjugated carbon:carbon bonds in the ring structure and have molecular weights in the range 500 to 1300. With few exceptions the biologic effects of these antibiotics have been attributed to the increased cellular permeability to a variety of solutes which results from the interaction of the polyene compounds with membrane bound cholesterol. This interaction is believed to be non-covalent and is dependent on the pH and relative concentrations of sterol and amphotericin (104). When amphotericin B is incorporated into a lipid film containing cholesterol, the sterol:phosphatidylcholine interactions are reduced allowing the surrounding phospholipid chains to undergo a normal, sharp, gel to liquid crystal phase transition as monitored by differential scanning calorimetry (DSC).



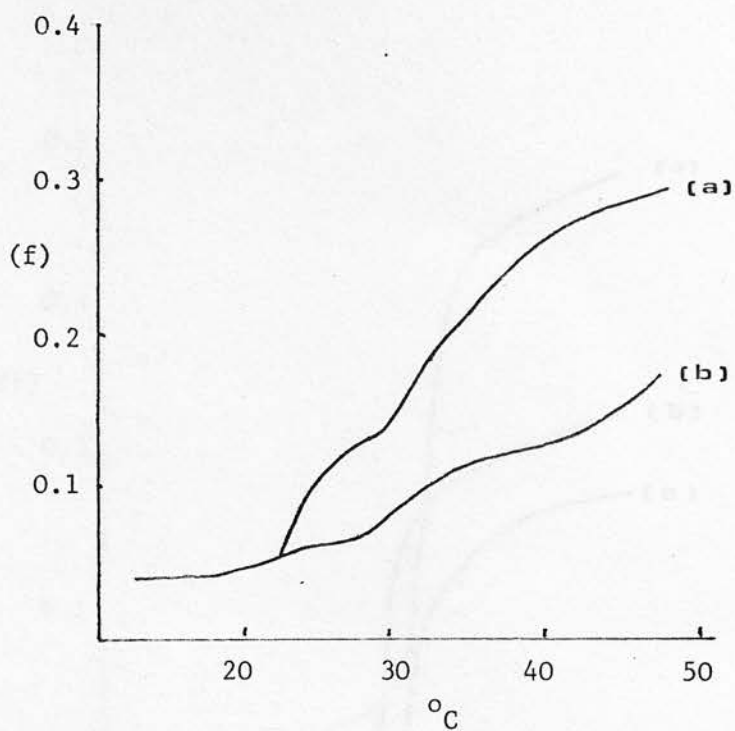
Tempo solubility in DPPC liposomes in the presence of (a) 1  $\mu$ l dimethyl-sulphoxide (b) Amphotericin B ( $0.3 \times 10^{-7}$  mole) in 1  $\mu$ l dimethyl sulphoxide (c) Amphotericin B ( $1.56 \times 10^{-7}$  mole) in 5  $\mu$ l dimethyl sulphoxide.

Fig. 4.18 (a)

Amphotericin B

Amphotericin B





Tempo solubility in DPPC liposomes  
containing (a) 20 mole % cholesterol  
(b) 20 mole % cholesterol +  $1.56 \times 10^{-7}$  mole  
Amphotericin B in 5  $\mu$ l dimethyl sulphoxide.

Fig. 4.18 (b)

Using Tempo solubility to monitor the phase transition of dipalmitoyl phosphatidylcholine instead of DSC I attempted to repeat the experiments designed to show that amphotericin B can remove cholesterol from phospholipid. The effect of varying concentrations of amphotericin on the phase transition of dipalmitoyl phosphatidylcholine in the presence and absence of 20 mole per cent cholesterol was measured (Fig. 4.18b).

As amphotericin B was extremely insoluble in dimethyl sulfoxide, which has no effect on Tempo solubility, was used to deliver the antibiotic to the liposome. Figure 4.18a shows that the addition of increasing amounts of amphotericin B to dipalmitoyl phosphatidylcholine had no effect on the sharpness of the phase transition but it did reduce the solubility of Tempo in the liquid crystal phospholipid. In addition the spin label method did not reveal any sequestration of cholesterol by amphotericin B as no fluidisation of phospholipid mixture was apparent. In fact the addition of amphotericin B seemed to reduce the solubility of Tempo and this was maybe due to the addition of the large rigid amphotericin B molecule.

#### 4.10 Solution interactions of cholesterol analogues and amphotericin B

The UV spectrum of amphotericin B recorded between 350 and 450 nm (104) and the effect of the addition of a ten fold excess of a cholesterol side chain analogue was noted. In each instance there was evidence of some kind of interaction between the respective cholesterol analogue and the antibiotic, which alters the ultra violet spectrum. This can be monitored by the change in the ratio of the peaks at 360 nm and 410 nm that occurs after a 30 minute incubation of amphotericin with sterol. These results are

Temp °C	Aqueous ( $\frac{N_i^{2+}}{A}$ )	Hydrophobic ( $\frac{N_i^{2+}}{H}$ )	( f )	$\frac{H^{N_i^{2+}}}{H}$	$\frac{A^{N_i^{2+}}}{A}$
35	4.4	1.2	0.214	0.719	0.406
38.5	3.9	1.7	0.304	0.711	0.404
43	2.55	3.8	0.598	0.710	0.409

The effect of  $8.4 \times 10^{-6}$  mole  $NiCl_2 \cdot 6H_2O$  on the aqueous and hydrophobic components of the Tempo spin label signal.

Temp °C	Aqueous ( $\frac{N_i^{2+}}{A}$ )	Hydrophobic ( $\frac{N_i^{2+}}{H}$ )	( f )	$\frac{H^{N_i^{2+}}}{H}$	$\frac{A^{N_i^{2+}}}{A}$
35	4.4	4.2	0.516	0.88	0.7
38.5	4.5	3.3	0.576	0.714	0.66
43	8.2	6.3	0.565	0.766	0.78

The effect of  $8.4 \times 10^{-6}$  mole  $NiCl_2 \cdot 6H_2O$  on the aqueous and the hydrophobic components of the 3N8 spin label signal.

Table 4.14

Note H represents the signal obtained in the absence of  $Ni^{2+}$

Cholesterol Analogue	ratio 360 / 410
C <sub>19</sub>	1.34
C <sub>21</sub>	1.34
C <sub>24</sub>	1.77
C <sub>25</sub>	2.17
C <sub>26</sub>	1.51
C <sub>27</sub>	1.73
C <sub>28</sub>	2.11
C <sub>29</sub>	1.82
E.Y.PC	0.9

pH 7 solution interactions of  $1.55 \times 10^{-7}$  mole  
cholesterol analogues with  $1.5 \times 10^{-8}$  mole  
amphotericin B.

Table 4.15

summarised in Table 4.15 which shows that although the interaction of amphotericin B with  $C_{19}$  and  $C_{21}$  was less than that with the longer side<sub>chain</sub> analogues, no absolute requirement for a specific side chain length was observed. This result was similar to the finding (105) that spectral changes of amphotericin B were not sensitive to the structure of a series of liposomally bound naturally occurring cholesterol analogues.

#### 4.11 Interactions of amphotericin B with liposomally bound cholesterol side chain analogues

Essentially the interaction was monitored in an identical manner to the solution interactions of amphotericin B and sterol described in the previous section. The liposomes were prepared from a dried lipid film containing 12 mole per cent ( $2 \times 10^{-8}$  mole), 25 mole per cent ( $5 \times 10^{-8}$  mole) and 33 mole per cent ( $1 \times 10^{-7}$  mole) cholesterol analogue by vortexing in 10 mM Tris-acetate at either pH 7 or pH 2. A dimethylsulphoxide solution of amphotericin B ( $1.5 \times 10^{-8}$  moles) was added to the liposomes and the mixture was incubated for 30 minutes prior to the recording of the U.V. spectra. Tables 4.16a,b do not give any indication of a sterol's side chain requirement for optimal interaction with amphotericin B molecules. Alteration of the proportions of antibiotic, sterol and egg yolk phosphatidylcholine had no effect on this interaction, but comparison with Table 4.14 showed that incorporation of the sterol in a liposome resulted in a reduction of the 360/410 ratio.

Unlike Norman et al. (104) the presence of 33 mole per cent cholesterol analogue did not seem to alter cholesterol:amphotericin B interaction at pH 2 and ~~7 and 10~~; the absolute results appeared to be similar at both pH values.

Sterol	mole% sterol		
	12 360/410	25 360/410	33 360/410
C <sub>19</sub>	1.33	1.38	1.48
C <sub>21</sub>	1.41	1.59	1.28
C <sub>24</sub>	1.52	1.74	1.79
C <sub>25</sub>	1.38	1.58	1.57
C <sub>26</sub>	1.42	1.69	1.48
C <sub>27</sub>	1.34	1.66	1.44
C <sub>28</sub>	1.34	1.84	1.44
C <sub>29</sub>	1.21	1.36	1.33
E.Y.PC	1.33	1.33	1.33

( a )

Sterol	mole% sterol		
	12 360/410	25 360/410	33 360/410
C <sub>19</sub>	1.55	1.44	1.28
C <sub>21</sub>	1.53	1.71	1.35
C <sub>24</sub>	1.59	1.72	1.37
C <sub>25</sub>	1.53	1.57	1.49
C <sub>26</sub>	1.65	1.52	1.35
C <sub>27</sub>	1.63	1.61	1.48
C <sub>28</sub>	1.48	1.53	1.30
C <sub>29</sub>	1.42	1.49	1.43

( b )

Interaction of amphotericin B (  $1.5 \times 10^{-8}$  mole )  
with cholesterol analogues contained in egg yolk  
phosphatidylcholine liposomes at (a) pH 7 and (b) pH 2

Table 4.16



Sterol	360/410 ratio	
	( a )	( b )
E.Y.PC	1.4	1.6
C <sub>19</sub>	1.3	1.2
C <sub>21</sub>	1.3	1.44
C <sub>24</sub>	1.8	1.35
C <sub>25</sub>	1.8	1.25
C <sub>26</sub>	1.8	1.05
C <sub>27</sub>	1.3	1.3
C <sub>28</sub>	1.69	1.2
C <sub>29</sub>	1.48	1.2

The effect of a 30 second sonication on the interaction at pH 7 of amphotericin B ( $1.5 \times 10^{-8}$  mole) with egg yolk phosphatidylcholine liposomes containing cholesterol analogues (  $2 \times 10^{-8}$  mole : 16 mole% sterol )

Table 4.17

The effect of sonication of amphotericin B and sterol containing liposomes is shown in Table 4.17. Column (a) shows the ratio 360/410 peaks after a 30 minute incubation of liposomes and amphotericin B. The mixture was then sonicated for 30 seconds and the ratio was remeasured, column (b). Although the sterol:amphotericin B interactions seems to withstand this sonication by comparison with the egg yolk phosphatidylcholine controls, the absolute values of the 360/410 ratio are reduced by approximately 20 per cent in all cases.

Taken together all the spin labelling experiments which were concerned with different aspects of the role of cholesterol in membranes show that cholesterol elicits the maximal response in each case. As a natural extension to the spin labelling experiments, the role of cholesterol was examined by a further set of physical techniques namely stopped-flow spectrophotometry and monolayer studies.

#### Summary to Chapter 4

- (1) AT 40 and 50 mole per cent cholesterol side chain analogue, cholesterol norcholesterol and sitosterol cause optimal ordering of the surrounding phospholipid chains as judged by the epr spectrum of 3NC incorporated into egg yolk phosphatidylcholine: analogue liposomes. With the rest of the available analogues the ordering is similar in all cases but the absolute value is less than that for cholesterol. A similar pattern is obtained with the 25NC spin label.
- (2) Oriented films of cholesterol analogue:egg yolk phosphatidylcholine (30:70) containing 1 mole per cent 3NC have slightly higher values for the order parameter as compared to liposomes of the same composition. The pattern observed is similar to the liposomes studies but the values for  $C_{28}$ , and  $C_{29}$  are intermediate between  $C_{27}$  and the other analogues.
- (3) Dipalmitoyl and dimyristoyl phosphatidylcholine liposomal studies with Tempo as an indicator group confirm the steroid spin label results obtained in egg yolk phosphatidylcholine although this spin label is reporting on a different physical effect. In addition use of the two phospholipids confirms that the sterol side chain and not the fatty acid side chain determines the cholesterol analogue phospholipid interaction.
- (4) The results of the 3 nitroxyl octane spin label studies are similar to those for Tempo although each spin label reports in a slightly different manner.
- (5) Interaction of the various spin labels with either ascorbate or nickel confirms that the 25NC spin label is buried deeper in the bilayer than the 3NC.

(6) Addition of amphotericin B in dimethylsulphoxide to an aqueous solution or a liposomal preparation of a sterol side chain analogue results in a sterol-amphotericin B interaction which is independent of the side chain present.

(7) Spin labelling using Tempo does not seem to be a useful technique for determining if amphotericin B can sequester cholesterol restoring the lipid phase transition in the process. Amphotericin B itself is a large, rigid, molecule and perhaps its presence interferes with the Tempo partitioning into the postulated cholesterol free regions in the dipalmitoyl phosphatidyl choline.

The implications of the results in Chapter 4 are discussed with the results of other physical studies, at the end of the following chapter.

## CHAPTER 5

### THE USE OF STOPPED FLOW SPECTROPHOTOMETRY AND MONOLAYERS TO STUDY THE INTERACTION BETWEEN CHOLESTEROL AND PHOSPHOLIPID

## 5.1 Introduction

The results described in the previous chapter were obtained with an external reporter group which partitions into a phospholipid:cholesterol dispersion. The possibility exists that the incorporation of the spin label into a membrane alters the structure of the membrane in such a way that the spin label's environment is unrepresentative of the bulk of the membrane. An alternative model system would be one in which the characteristics of the membrane could be determined in the absence of a reporter molecule. In this chapter two further physical techniques are described (a) stopped flow spectrophotometry of liposomes, and (b) monolayer studies.

### (a) Stopped flow spectrophotometry of liposomes

Liposomes formed by swelling a dried lipid film in an aqueous salt solution consist of concentric spheres of lipid bilayers whose precise geometry depended on the water content, temperature, ionic strength and the method of dispersion (25). At equilibrium each and every lipid bilayer was visualised (106) as forming an unbroken membrane implying that every aqueous compartment was discrete and isolated from its neighbour. In addition the liposomally bound aqueous compartment was separated from the bulk aqueous phase in which it was suspended. Electron microscopy of liposomes (107) showed this closed structure was present and revealed that each biomolecular lamella was separated by  $45\text{\AA}$  by an aqueous compartment, which contained water and trapped ions from the swelling buffer. This intraparticulate water can be lost by the addition of an osmotically active species such as hypertonic salt solution.



77.

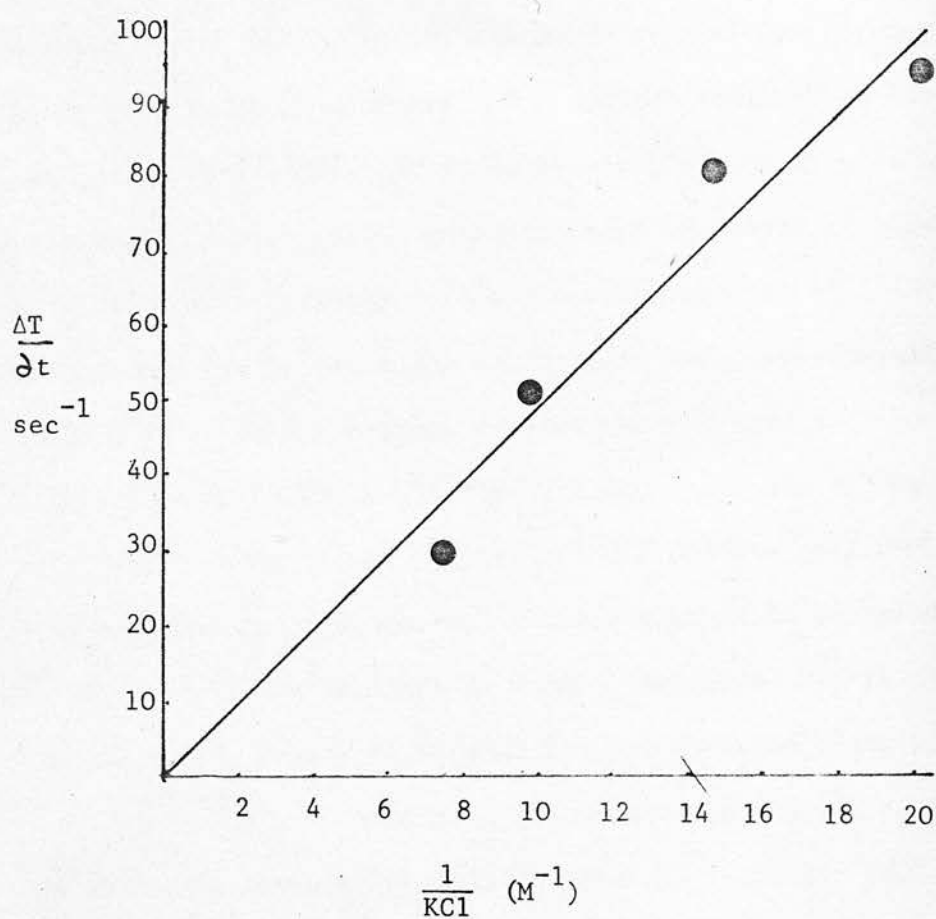
The permeability of liposomes has been monitored spectrophotometrically in a stopped flow device by Bittman and Blau (108) and Jain et al. (26). They assumed that the amount of light scattered by liposomes was a function of their volume at those wavelengths where absorbance was due entirely to scattered light (109). In addition the liposomes were shown to be ideal osmometers and thus were permeable to water but not to solute molecules. The net flow of water through a membrane per unit time is proportional to the surface area of the membrane and the osmotic gradient across it and the large surface area of liposomes permits the initial rate of volume change upon sudden change in the osmotic pressure of the suspending medium to be measured. With potassium chloride in the osmotically active spaces the initial rate of the volume change provided an indication of the velocity of water diffusion through the outer lipid layer. Dicetyl phosphate was included in some preparations (108) in order to confer a net negative charge on the bilayer which increased the liposomal radius facilitating the trapping of potassium chloride in the aqueous interstices.

In recent years two main molecular mechanisms have been proposed for water transport across lipid bilayers. In the first mechanism, non-specific water permeation is explained in terms of channels or pores within the membrane which are transiently formed by thermal fluctuations of the molecules comprising the membrane structure (110). Electron microscopy of liposomes does not reveal the existence of such tiny pores and thus this explanation of the phenomenon has become less popular.

The second and more important theory of transmembrane movement is that proposed by Träuble (111). Transmembrane movement of

water depends on the partition coefficient of the permeating molecules between the aqueous phase and the hydrocarbon region of the membrane. The relationship of both phases can be altered by thermal fluctuations in the hydrocarbon chains of membrane lipids. The thermal fluctuations result in the formation of conformational isomers which are the so-called "kink" isomers of the hydrocarbon chains. The kinks so formed may be described as mobile structural defects which represent small mobile free volumes of different size depending on the type and arrangement of kinks in the hydrocarbon membrane phase. A molecule present in the aqueous phase adjacent to the membrane may jump into the free volume of a kink at the membrane surface and may then diffuse across the membrane together with the mobile kink in a rapid hitch-hiking process ( $10^{-5} \text{ cm}^2/\text{sec}$ ). By an appropriate combination of kinks volumes large enough to accommodate molecules of water can be formed. Most of these holes will only extend for a few chain segments and not completely across the membrane so that in general no pores would be formed.

The effect of cholesterol on the liposomal structure has been well documented (100). Johnson (112) has shown that the addition of 10 mole per cent cholesterol resulted in a 30% increase in the phospholipid molecule surface area with a consequent decrease in bilayer thickness. As the molar proportion of cholesterol rose further the surface area of the bilayer per phospholipid molecule remained constant at  $89\text{\AA}^2$  and the bilayer thickness increased. In addition the X-ray data (22) obtained for dipalmitoyl phosphatidylcholine liposomes revealed that there was a large increase in the long spacing of the X-ray diffraction bands from  $70\text{\AA}$  to  $81\text{\AA}$  in the presence of 7.5 mole % cholesterol and a decrease to  $64\text{\AA}$  at 50



Demonstration that the liposomes are behaving as ideal osmometers.

Fig. 5.1

mole % cholesterol.

Both of these observations correlate with the effect that varying proportions of cholesterol have on water permeability across lipid bilayers. Jain et al. (26) have shown that water permeability increased up to 10 mole % cholesterol and decreased beyond this level of sterol. Their results can be explained by the relative abundance of kinks present in each mixture due to the different packing characteristics of phospholipids in the presence of different amounts of cholesterol.

In order to clarify the cholesterol mediated reduction in permeability, it was decided to determine what effect altering the side chain structure of cholesterol had on water permeability across a bilayer.

#### 5.7 Osmotic shrinking of liposomes prepared in 0.05 M KCl

The liposomes were prepared as described in the Materials and Methods section. Figure 5.1 shows that liposomes prepared with potassium chloride as the osmotically active species give linear Boyle-van't Hoff characteristics for osmotic shrinking when exposed to an osmotic pressure gradient. The linear dependence observed for liposomal osmotic shrinking indicates that they are ideal osmometers under these conditions and are completely impermeable to solute, and thus they are ideal for the study of the effect of the incorporation of cholesterol analogues with altered side chains.

As mentioned in the previous section, liposomes prepared for permeability studies either contain phosphatidic acid (109), or dicetyl phosphate (26,108). The absolute amount of dicetyl phosphate used by other workers varies from 20 mole per cent (108)

Fig. 5.2 (a)

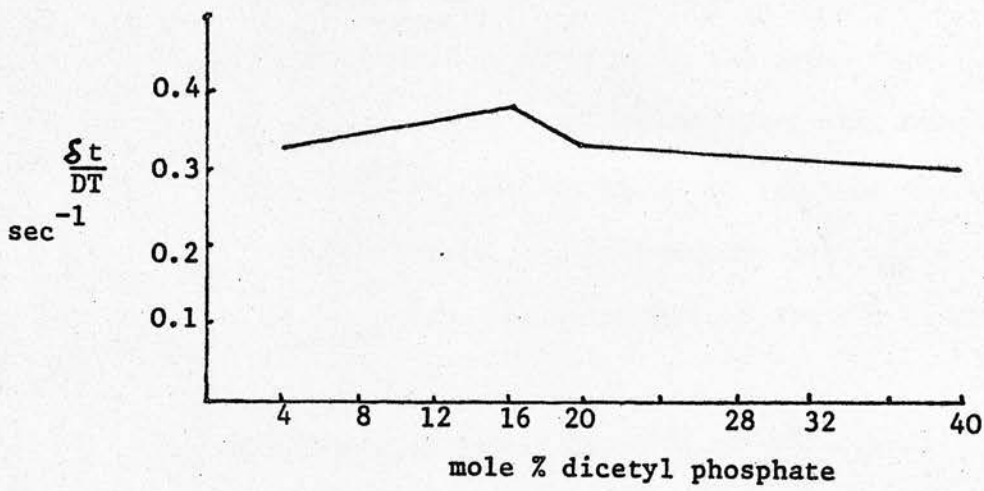


Fig. 5.2 (b)

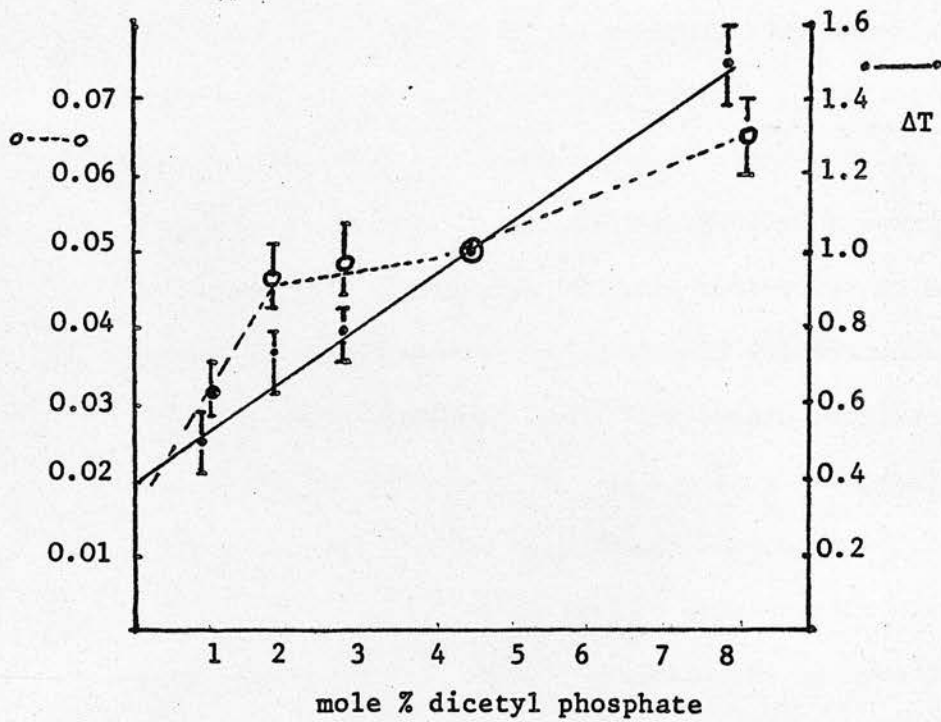


Fig. 5.2 (a)

The effect of increasing mole % dicetyl phosphate on the ratio of  $\delta t$  to  $\Delta T$ .

Fig. 5.2 (b)

The effect of increasing mole % dicetyl phosphate on  $\delta t$  and  $\Delta T$ .



to 4 mole per cent (26). At either of these concentrations the presence of dicetyl phosphate increases both the total extent of shrinking ( $\Delta T$ ) and the initial rate of shrinking ( $\partial t$ ), Fig. 5.2b. In order to ensure that any effect observed was due to the presence of sterol and not to the presence of dicetyl phosphate, Jain et al. (26) calculated the ratio of the initial rate of shrinking to the total extent of shrinking,  $\partial T/\Delta T$ , and it is their approach that is employed in this chapter. Figure 5.2a shows that even up to 40 mole per cent dicetyl phosphate,  $\partial t/\Delta T$  remains constant in dicetyl phosphate:phospholipids liposomes. Thus any alteration in this value ( $\partial T/\Delta T$ ) cannot be attributed to variation in dicetyl phosphate present when the data are treated in this manner.

### 5.3 The effect of cholesterol analogues on water permeability across egg yolk phosphatidylcholine liposome membranes

The effect of cholesterol on water permeability of phospholipid bilayers has been documented by Bittman and Blau (108) and Jain et al. (26). Both groups of workers studied the osmotic shrinking in a stopped flow spectrophotometer which allows measurement to be made almost instantaneously as opposed to the 2-5 second delay experienced with mechanical mixing (109). Bittman and Blau found that cholesterol decreased the initial rate of volume change by 42% at 33 mole per cent cholesterol and by 30% at 20 mole per cent cholesterol. In addition they found that introduction of unsaturations into the hydrocarbon fatty acid chain increased water permeability across the liposomal membrane. Taken together these results appear to be consistent with the solubility diffusion mechanism for passive diffusion of water. Cholesterol could lower the

Time (secs)	Measured difference to end point	$\log x_t$	$\log x_t/x_0$
0.	6.9	0.839	0
0.1	5.6	0.748	-0.091
0.2	4.8	0.681	-0.158
0.3	4.5	0.653	-0.186
0.4	4.2	0.623	-0.216
0.5	3.85	0.585	-0.254
0.6	3.8	0.580	-0.259
0.7	3.5	0.544	-0.295
0.8	3.4	0.531	-0.308
0.9	3.2	0.505	-0.334
1.0	3.0	0.477	-0.362

From figure 5.3 the slope of  $\log(x_t/x_0)$  versus  $t$  is

$$\log x_t - \log x_0 \text{ per second} = -0.245$$

Converting to a positive mantissa

$$\log x_t - \log x_0 \text{ per 1 second} = 1.755$$

Since  $\log x_0$  is 0.839

$$\log x_1 = 0.839 + 1.755 = 0.594$$

Antilog:  $x_1 = 3.93$

Therefore, in 1 second there has been a transmittance

change of  $6.9 - 3.93 / 20 \times 0.5 \text{ V} = 0.0745 \text{ V}$  if  $1 \text{ cm} = 0.025 \text{ V}$

$$\delta t = 0.0745 \text{ V}$$

$$\Delta T = 0.173 \text{ V change in 50 seconds}$$

$$\delta t / \Delta T = 0.430 \text{ sec}^{-1}$$

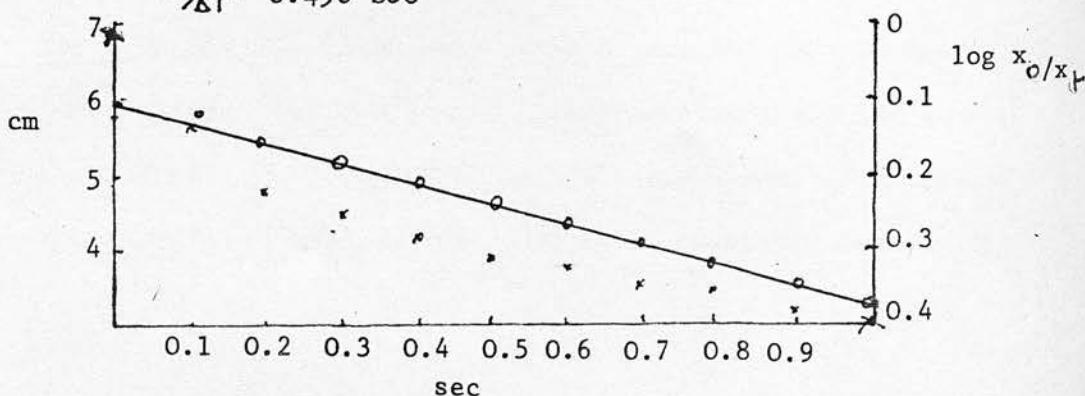


Fig. 5.3

An example of the method of calculation of the kinetic parameter,  $\delta T/DT$ , from the raw data collected by the transient recorder.

diffusion coefficient of water permeation by rigidifying the crystalline hydrocarbon chains and limiting the formation of mobile structural defects. The reduction in permeability was not so marked when the OH group at position 3 was changed from  $\beta$  to  $\alpha$  (epicholesterol) or replaced by an SH group (thiocholesterol). Thus there appeared to be a precise structural requirement of the polar groups for optimal cholesterol-phospholipid interaction.

In order to determine if alterations of the side chain structure of cholesterol affected its ability to reduce water permeability, the available cholesterol analogues were incorporated into egg yolk phosphatidylcholine liposomes. At the concentration used, all the analogues including cholesterol can be equally incorporated into a liposomal structure. Essentially the experimental method employed was that described by Jain et al. (26). As described by Bittman and Blau (108), use of a 1:1 ratio of liposomes and 0.18 M KCl occasionally resulted in a disturbance of the trace within the first 150 m secs after mixing. For each liposomal mixture the initial rate of osmotic shrinking over the first second was calculated from the exponential curve by treating the transmittance data in the manner (113) described in Figure 5.3 and expressing it as a fraction of the total transmittance change.

The results of the osmotic shrinking experiments are given in Table 5.1 and 5.2 which show that the values for different batches of phosphatidylcholine and different liposomal preparations can vary by up to 25 per cent, although the normal variation is approximately 10 per cent. This divergence is similar to figures quoted by Jain et al. and may be due to

mole% sterol	C <sub>19</sub> $\delta T/\Delta T$	C <sub>21</sub> $\delta T/\Delta T$	C <sub>27</sub> $\delta T/\Delta T$
0	0.463	0.602	0.629
10	0.463	0.602	0.492
20	0.463	0.602	0.478
30	0.463	0.580	0.410
40	0.500	0.570	0.383
50	0.353	-----	0.342

Table 5.1

Comparison of  $\delta T/\Delta T$  (22°C) for liposomes containing C-50 mole% C<sub>19</sub>, C<sub>21</sub>, and C<sub>27</sub>. Results are the mean of at least 4 experiments.

mole% sterol	$\delta T/\Delta T$							
	C <sub>19</sub>	C <sub>21</sub>	C <sub>24</sub>	C <sub>25</sub>	C <sub>26</sub>	C <sub>27</sub>	C <sub>28</sub>	C <sub>29</sub>
0	0.463	0.602	0.578	0.620	0.580	0.629	0.472	0.540
10	0.463	0.602	0.578	0.530	0.548	0.492	0.451	0.532
33	0.463	0.580	0.472	0.510	0.408	0.410	0.454	0.525

Table 5.2

The effect of cholesterol analogue incorporation on water permeability across the liposomal membrane at 22°C. The results are the mean of at least 4-5 experiments.

Temp. (°C )	C <sub>19</sub>	C <sub>21</sub>	C <sub>24</sub>	C <sub>25</sub>	C <sub>27</sub>	C <sub>28</sub>	C <sub>29</sub>
25	0.96	1.07	0.91	0.89	0.69	1.03	1.07
30	0.96	0.92	0.94	0.88	0.72	1.05	0.98
35	0.91	0.93	0.89	0.89	0.88	1.02	0.91
40	0.98	0.96	/	1.00	0.97	1.01	0.98
45	0.97	0.97	0.93	0.99	0.88	1.05	0.90

The effect of temperature on water permeability across liposomal membranes containing 25 mole<sup>%</sup> cholesterol side chain analogue. Results are the mean of 2-4 independent experiments.

Table 5.3



differences in the numbers of sealed vesicles present from batch to batch. As shown in Table 5.1 even at 40 mole per cent sterol,  $C_{19}$  and  $C_{21}$  sterols do not result in a reduction of the water permeability, whereas cholesterol causes a 39% decrease.

Table 5.2 gives the values for  $\partial T/\Delta T$  at 0, 10 and 33 mole % sterol, and reveals that the incorporation of 33 mole per cent sterol results in a 17% reduction in permeability in the case of  $C_{24}$  and  $C_{25}$  and a 29 and 35% reduction in liposomes containing  $C_{26}$  and  $C_{27}$  respectively. When  $C_{19}$ ,  $C_{21}$ ,  $C_{28}$  and  $C_{29}$  are present, no reduction in permeability is observed.

#### 5.4 Temperature effects on water permeability across liposomal membranes containing cholesterol analogues

The effect of increasing temperature on water permeability is detailed in Table 5.3. At 25°C a similar trend to that described in the previous section is observed.

At 35°C the differences between the analogues are eliminated and this trend is continued at 40°C and 50°C. The  $\partial T/\Delta T$  results in the table at each temperature are expressed as the ratio of the value for egg yolk phosphatidyl choline due to the previously mentioned variability in the phosphatidylcholine preparations and are calculated from an average of four determinations on each occasion.

The main conclusion of Sections 5.3 and 5.4 is that altering the side chain of cholesterol by the removal or addition of methyl groupings results in the diminution of the regulatory role that cholesterol plays in water permeation.

Increasing the temperature to 35°C removes the cholesterol mediated reduction of water permeability noted at 25 mole per cent sterol. This observation can be easily explained by Träuble's

theory of water permeation (111) in terms of the increased likelihood of kink formation at elevated temperature. The increased number of kinks present could alter the liposomal structure.

Thus the water permeability characteristics of sterol:phospholipid liposomes are comparable with sterol free liposomes on increasing the temperature.

## 5.5 Monolayer studies of cholesterol:phospholipid interactions

When a chloroform solution of lipid is placed on the surface of a water layer in a Langmuir trough by means of microsyringe, the solvent evaporates leaving insoluble lipid monolayers at the air water interface. The film so formed can then be made to compress by means of a moveable barrier in contact with the film, until it collapses. The area per molecule for a monolayer can be calculated at any surface pressure. Hence the monolayer studies permit a direct determination of the cross-sectional area of an insoluble surface-active molecule. Usually the results are shown by a surface pressure-area ( $\pi$ -A) plot. Surface pressure can be regarded as the osmotic pressure of a two dimensional solution.

When cholesterol was added to phospholipid preparations the mixed monolayers were considerably condensed, and the area per molecule was less than the sum of each individual molecular area (114). An indication of the precise role played by the phospholipid in the cholesterol:phospholipid mixture was provided by Demel et al. (115), by preparing, among others, mixed monolayers of 1,2 dimyristoyl phosphatidylcholine and cholesterol, and 1,2 distearoyl phosphatidylcholine and cholesterol. The mixed monolayers of cholesterol and 1,2 distearoyl phosphatidylcholine essentially followed the additivity rule of molecular areas, whereas

cholesterol caused condensation of 1,2 dimyristoyl phosphatidylcholine. Thus a slight alteration of the fatty acyl chain was seen to have an important effect on the monolayer cholesterol phospholipid interaction.

The same group have also studied the structural requirements for sterols for sterol:phosphatidylcholine interaction at the air water interface (89). Using a selection of  $3\alpha$  and  $3\beta$  hydroxy sterols, and 3 keto-steroids, the interaction of sterol with 18:1/18:0 phosphatidylcholine in a 1:1 monolayer was determined by calculation of the area per molecule at  $22^{\circ}\text{C}$ . On the basis of their monolayer data it could be demonstrated that cholestanol, lathosterol, 7-dehydrocholesterol and  $\beta$ -norcholesterol were as effective as cholesterol in achieving a minimal mean molecular area (Fig. 1.1). The mixture of 18:1/18:0 phosphatidylcholine with the plant sterols ergosterol and stigmasterol, and with androstanol showed a slightly higher mean molecular area. The less effective interaction of these three compounds must be due in the cases of ergosterol and stigmasterol to double bond at  $\text{C}_{22}$ ,  $\text{C}_{23}$  and the extra methyl and ethyl group respectively at  $\text{C}_{24}$  and to the lack of an intact side chain in the case of androstanol.

The apparently complex behaviour of various phosphatidylcholine:sterol monolayers has been interpreted in two different ways

(a) One interpretation attributes the observed effects to alteration in Van der Waals interactions, configuration entropy effects and alterations in the water structure adjacent to the monolayer.

(b) The second interpretation proposed by Shah and Shulman (23)

stated that the deviation in molecular area was not due to an interaction, but rather to steric factors such as unsaturation and length of phosphatidylcholine hydrocarbon chains. They showed that the average area per molecule in dipalmitoyl-phosphatidylcholine:cholesterol monolayers exhibited deviation at low surface pressure but at 30 dynes/cm the monolayers followed the additivity rule.

The apparent condensation of the mixed monolayers was explained as follows.

The fatty acyl chains of phosphatidylcholine in an expanded monolayer because of their thermal motion effectively occupy a volume represented by a cone with its apex at the air water interface and base at the methyl terminal of the fatty acyl chains. This movement of fatty acid chains would result in the formation of a vacancy or cavity between molecules. If other molecules that have dimensions smaller than or equal to those of the cavity are added to the phosphatidylcholine monolayer they may partially occupy these cavities without causing a proportional increase in the area of the monolayer. This would explain the observed deviation from the average area per molecule (the additivity rule). At high surface pressures the fatty acyl chains are vertically packed and the addition of cholesterol therefore causes a proportional increase in the area of the mixed monolayer.

Shah and Shulman (23) have also shown that egg yolk phosphatidylcholine:cholesterol monolayers are optimally condensed at molar ratios of 1:3 and 3:1 which allow optimal geometrical arrangements of those molecules for packing in the mixed monolayer. This ratio is thought to be related to the fraction of oleoyl chains in the total fatty acyl chains of egg yolk phosphatidylcholine.

Fig. 5.4

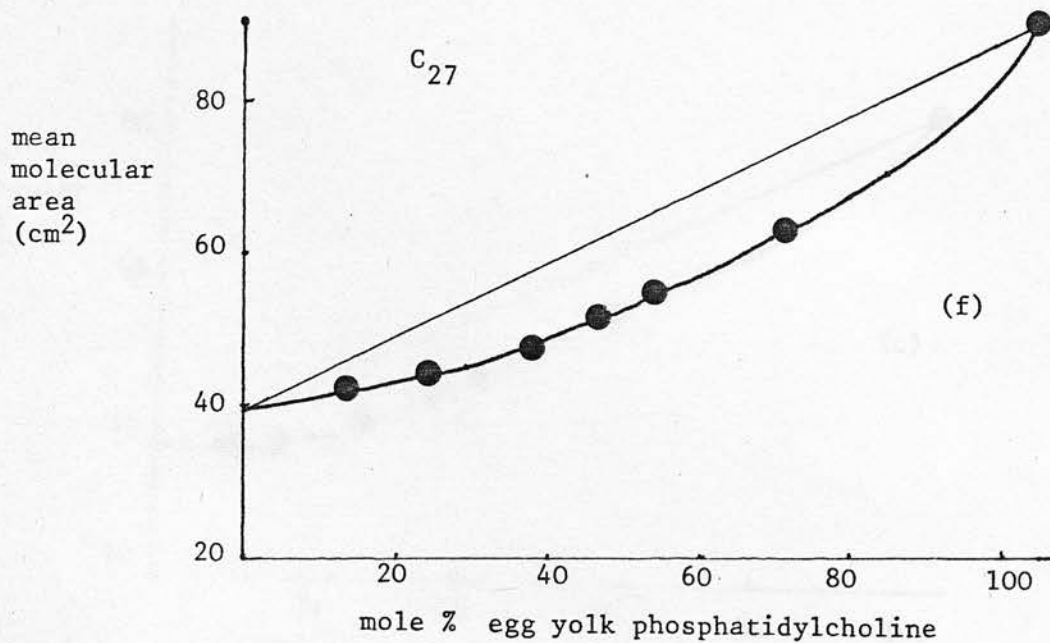
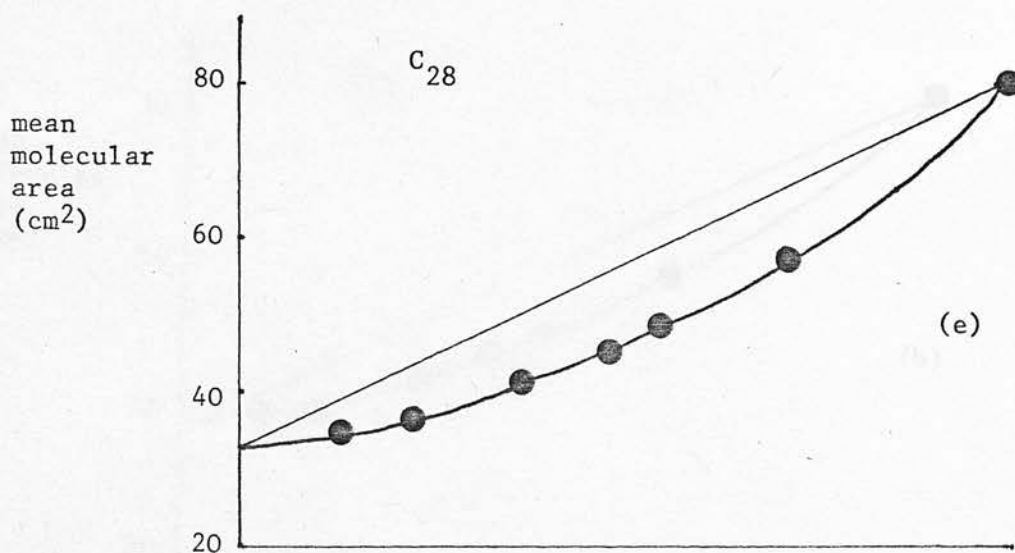
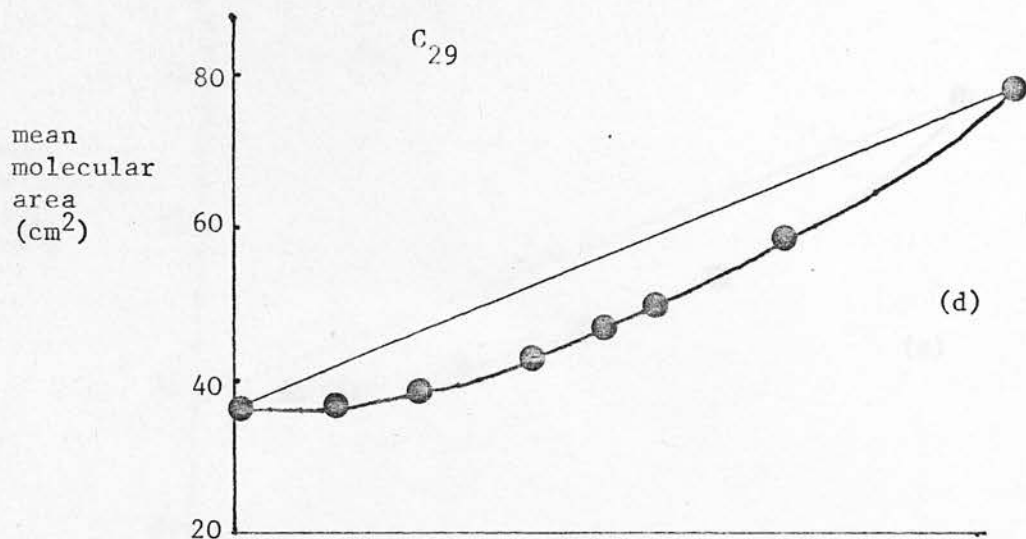


Fig. 5.4

A comparison of the effect of the incorporation of a series of cholesterol side chain analogues on the mean molecular area of mixed monolayers.



The monolayer packing characteristics of the available cholesterol side chain analogues were assayed and the results of these experiments were related to the spin label and the osmotic shrinking results.

#### 5.6 Measurement of the force area curve for mixed films of cholesterol analogues and egg yolk phosphatidylcholine

These experiments were performed by Dr. B.R. Malcolm, Department of Molecular Biology, University of Edinburgh.

A quantitative measurement of the cholesterol analogue's interaction with its surrounding lipid molecules was obtained by plotting the variation of the mean molecular area at a constant surface pressure (12 dynes/cm) as a function of the egg yolk phosphatidylcholine mole fraction present. This is the treatment employed by Goodrich (see 116) and the results are plotted in Figure 5.4a-f. Analysis of the data at 50 mole per cent sterol shows that  $C_{19}$  and  $C_{21}$  exhibit a 12% deviation from ideality, whereas all the other analogues tested exhibited approximately a 20% deviation from ideality.

The results show that monolayer interaction between cholesterol and side chain analogues and egg yolk phosphatidylcholine does not have the absolute requirement for an isoctane side chain for optimal interaction previously observed in the liposomal studies.

Perhaps the lack of specificity in monolayers can be explained by the absence of the constraints imposed on liposomal cholesterol phospholipid interactions as a result of incorporation into a bilayer structure of fixed dimensions. Thus the variation in the cholesterol side chain seems to assume a greater importance with regard to cholesterol's molecular packing properties when the phospholipid-cholesterol interaction is subjected to certain

structural limitations. These structural limitations also enable cholesterol to exert a molecular control of membrane morphology in areas with high radius curvature (117). Such changes in lateral distribution of cholesterol (69) could result in alteration of the biological activity of the cholesterol containing membrane.

A discussion of the role of the cholesterol side chain in the interaction between cholesterol and phospholipids

The foregoing two chapters have been concerned with trying to define the role played by the cholesterol side chain in the interaction between cholesterol and phospholipids when both are incorporated into a model membrane.

Chapter 4 dealt with the epr studies of the interaction using a variety of spin labels. Although the four spin labels were reporting on slightly different aspects of the interaction they all showed the same basic trend. The results obtained showed slight differences from label to label. The 3NC spin label reported on the effect that the incorporation of cholesterol side chain analogues had near the liposome surface whereas the 25NC spin label reporter group was situated deeper in the membrane structure. Both spin labels showed that cholesterol has the optimal structure for sterol-phospholipid interaction but the absolute values differ in such a way as to suggest that cholesterol exerts its maximal ordering effect at the bilayer surface. At 50 mole per cent cholesterol, the 3NC order parameter showed a 69% increase over that observed with egg yolk phosphatidylcholine alone, whereas the 25NC order parameter indicated that 50 mole per cent cholesterol showed a 55% increase in ordering. As the 3NC spin label signal is more rapidly removed by ascorbate than <sup>the</sup> 25NC signal the results

are consistent with the hypothesis that cholesterol is exerting its maximal ordering effect nearer the phospholipid head group region than the bilayer centre. In addition the 25NC results showed that incorporation of 10 per cent sterol caused an ordering of the bilayer centre whereas the 3NC did not detect any change in fluidity of phospholipid at this sterol concentration. Thus both spin labels gave the same overall picture but there were differences in the actual values obtained.

The preparation of the oriented sterol:egg yolk phosphatidylcholine films containing 3NC which allowed a greater resolution of the spectra also showed that cholesterol caused the maximum increase in order parameter. The values of the order parameter calculated for oriented films are higher due to the separation of the various spectral contributions that are averaged in the 3NC liposomal spectra.

When Tempo and 3N8 were used to report on the cholesterol interaction with defined, saturated, phosphatidylcholine, the same basic pattern was noted with cholesterol causing the maximum broadening of the phase transition at all the concentrations studied. The results for the  $C_{19}$  analogue monitored by 3N8 were slightly unusual in that there was no deviation from ideality up to 30 mole per cent sterol. In the other experiments at all concentration the  $C_{19}$  analogue caused a moderate increase in rigidity with the  $C_{28}$  and  $C_{29}$  somewhere between  $C_{19}$  and cholesterol. The simple act of removing the iso-octane side chain completely or adding to its length obviously has an important effect on sterol phospholipid interactions studied by spin labels.

These last observations can be explained in one of two ways

(a) In terms of a cleft formed between the phospholipid molecules in a bilayer which is optimally adapted to fit the cholesterol molecule.

(b) By considering that the phospholipid fatty acyl chains exist in an equilibrium between an ordered state and a state of random motion. Their precise equilibrium position is determined by the structure of the cholesterol analogue that is incorporated into the phospholipid bilayers.

(a) The existence of a cleft of precise dimensions, specific for the cholesterol side chain, between the phospholipid molecules is an attractive proposition which would explain the results obtained with cholesterol molecules with altered side chains. The cleft would only be formed when the concentration of cholesterol analogues was increased to levels where it started to rigidify the fatty acyl chains. At approximately 30 mole % sterol the cleft would be visualised as exerting a fine adjustment on cholesterol-phospholipid interaction by its differing interaction with the cholesterol side chain of various lengths. The cholesterol analogues with shorter side chain than cholesterol have a higher motional freedom as cleft is only partially occupied. This increase in the motion of the analogue would be observed through the bilayer with a consequent decrease in the order parameter as compared to the molecule with the optimal fit in the cleft (cholesterol). The decrease in ordering of the spin labels observed with the sterols with longer side chains  $C_{28}$  and  $C_{29}$  could also be explained in terms of the existence of a precise cleft that is adapted for optimal fitting by an iso-octane side chain. If the sterol nucleus of  $C_{28}$

or  $C_{29}$  was positioned in a phospholipid bilayer in an identical manner to cholesterol (49) then especially in the case of  $C_{29}$  the larger side chain would protrude through the other half of the bilayer. However if the existence of a cleft with specific limitation regarding the size of a side chain that it can accommodate is postulated, incorporation of longer side chain analogues would result in altered packing characteristics of their ring system. This alteration of the molecular packing would also reduce the interaction by the cholesterol hydroxyl group with the phospholipid head group (51) by increasing their spatial separation. The increase in disorder of the bilayer caused by the disruption of the specific interaction normally operative in the case of cholesterol is consistent with the calculated order parameters.

(b) In the absence of sterol the fatty acyl chains of egg yolk phosphatidylcholine are in rapid motion and loosely packed. This loose association allows molecules of cholesterol to be intercalated between the fatty acyl chains which disrupts the intermolecular associations operative in the phospholipid. As the molar proportions of cholesterol present are increased the available sites that cholesterol can occupy decrease in direct proportion. This has the dual effect of increasingly immobilising the fatty acid chains and altering the equilibrium that existed between the rigid and fluid fatty acyl chains. The equilibrium position of that membrane can be slightly altered by the selective addition or subtraction of carbon atoms from the side chain of the cholesterol molecule. The short side chain analogues may allow more freedom of movement of the surrounding phospholipid molecules and thus the order of the bilayer is decreased as compared with the



ordering of phospholipid by an equivalent amount of cholesterol. In the case of  $C_{28}$  and  $C_{29}$  where the side chain is extended, the order of the sterol:phospholipid bilayer is decreased which is similar to the results obtained with the short side chain analogues. A possible explanation for this disordering effect could be that in the case of  $C_{28}$  and  $C_{29}$  the side chain will penetrate into the other half of the bilayer and increase the disorder. Alternatively, the side chain could fold back on itself, thus occupying a greater volume than the cholesterol side chain, leading to the increase in the movement of the fatty acyl chains.

Explanations (a) and (b) partly account for the observed specificities for cholesterol analogue phospholipid interaction. Perhaps each new equilibrium position established by the addition of varying amounts of cholesterol analogue result in the formation of transitory clefts between the fatty acyl chains which are optimally adapted to fit the cholesterol molecule. The work of Opella et al. (45) has shown that the motion of the isopropyl tail of the cholesterol side chain is subject to steric restraints and thus this would tend to suggest that some interaction is occurring between the sterol side chains and its surroundings. The overall results of Chapter 4 suggests that alteration of the side chain interferes with this interaction.

Chapter 5 described the cholesterol-phospholipid interactions using two physical techniques which did not require the addition of an external reporter group. The results of the osmotic shrinking experiments at  $25^{\circ}\text{C}$  showed that at 10 mole % sterol none of the cholesterol side chain analogues (including cholesterol itself) cause a reduction in water permeability. At 33 mole %



the effect of sterols with altered side chains became apparent, with  $C_{19}$ ,  $C_{21}$ ,  $C_{28}$  and  $C_{29}$  causing no reduction in permeability and  $C_{26}$  and  $C_{27}$  reducing the permeability by 29 and 35%. The observed value for  $C_{24}$  lay exactly in the middle of these two groups (17% reduction). These results can be simply explained by Trauble's theory (111) on the grounds that alteration of side chain will interfere with the optimal packing described in Chapter 4, resulting in the formation of free volumes in the bilayer capable of holding water. The fact that increasing the temperature to  $35^{\circ}\text{C}$  abolished these side chain differences and the cholesterol mediated reduction in water permeability, indicated that an increase in the thermal energy of the fatty acids could overcome the rigidifying effects of cholesterol at this concentration allowing the water to permeate freely.

A similar set of experiments using *Acholeplasma laidlawii* membranes showed that while cholesterol increased the molecular packing, decreased the fluidity of phospholipid hydrocarbon chains and decreased non-electrolyte permeability, epicholesterol had none of these effects and cholesterol had opposite effects to epicholesterol (29,118). It has been concluded that a planar steroid nucleus, an intact side chain bearing no polar groups and a  $3\beta$  hydroxy group are needed for the permeability reducing effect, and these are the same factors that were required to induce optimal order of spin probes in hydrated films of beef brain phospholipid (95). In contrast Singer and Wan (119) reported that there was no apparent correlation between the effects of various steroids on the permeability of egg yolk phosphatidylcholine-dicetyl phosphate liposomes to  $\text{Na}^{+}$  and the fluidity as measured from the epr spectra of spin labels intercalated in a lipid dispersion. This report

seems to contradict the more recent results of Butler and Smith (120) which were consistent with their earlier conclusions (95) that sterols which disorder lipid bilayers (epicoprostanol) lead to increased permeability whereas those which order lipid bilayers lead to decreased permeability (cholesterol and cholestanol).

Thus the results of the spin labelling experiments and the osmotic shrinking studies using liposomes containing cholesterol analogues with altered side chains confirmed the correlation between spin labelling and osmotic shrinking results observed by other workers. Furthermore they showed that the effect of cholesterol on both processes was closely linked with the iso-octane side chain structure.

The other half of Chapter 5 dealt with cholesterol phospholipid interactions in a monolayer. The results did not show the same pattern that had been previously observed in the other physical methods.  $C_{19}$  and  $C_{21}$  showed a 12% deviation from ideality whereas the rest caused a 20% deviation.

An increase in the side chain beyond eight carbons does not decrease the interaction of cholesterol analogue with the phospholipid.

The monolayer results can be explained in terms of the removal of a constraint on the interaction between cholesterol and phospholipid that operates in the liposomal system. Evidence for this constraint comes from the reported alterations in the packing characteristics of cholesterol in liposomes as the proportion of cholesterol is increased (19). The incorporation of cholesterol in a bilayer reveals certain structural modifying properties of cholesterol which are not observed when it is present in a monolayer of identical composition.

In conclusion, the results of the liposome studies show that when the cholesterol phospholipid interaction is monitored by a series of techniques the important role played by the side chain in regulating this interaction becomes apparent. In the intact cell the cholesterol side chain may be performing a similar function to that observed in models, but this system is more difficult to evaluate due to the presence of heterogeneous phospholipids and different classes of proteins which complicate the interpretation of the data.

Preliminary experiments which extend this study to natural membranes are described in Chapter 7.

## CHAPTER 6

### OXIDATIVE METABOLISM OF THE CHOLESTEROL ANALOGUES BY THE CHOLESTEROL OXIDATION ENZYME SYSTEMS IN RAT LIVER MICROSOMES AND RAT ADRENAL MITOCHONDRIA

## 6.1 Introduction

Two of the main sites of cholesterol metabolism are located in liver endoplasmic reticulum <sup>and</sup> in adrenal cortical mitochondria. These are the respective locations of cholesterol 7 $\alpha$ -hydroxylase which is thought to be the rate limiting step in the production of bile acids from cholesterol, and the cholesterol side chain cleavage enzyme which converts cholesterol to pregnenolone.

The cholesterol 7 $\alpha$ -hydroxylase enzyme is a cytochrome P450 dependent enzyme as is the drug metabolising enzyme system also found in the liver. In this chapter several experiments have been performed to find out the substrate specificity of both enzymes and the effect of various treatments on cholesterol 7 $\alpha$ -hydroxylase activity in order to determine if cholesterol 7 $\alpha$ -hydroxylation was achieved by the drug metabolising enzymes.

The drug hydroxylation system of rat liver microsomes accepts a remarkable range of substrates, enabling compounds as diverse as ethylmorphine, aminopyrine, cyclohexane, and aniline to be hydroxylated. Conversely the cholesterol 7 $\alpha$ -hydroxylase enzyme seems to have definite structural requirements for substrate molecules. These structural features have been investigated by presenting a series of cholesterol analogues in acetone to the enzyme and noting their metabolic fate. Brown and Boyd (2) showed that androst-5-en-3 $\beta$ -ol-17-one, androst-5-en-3 $\beta$ -ol, and pregn-5-en-3 $\beta$ -ol were metabolised to one or more products when they were incubated with rat liver microsomes, a source of NADPH, and a molecular oxygen. This pattern of metabolism was unaffected by cholestyramine. This study was repeated in part (10) but in addition a more comprehensive collection of cholesterol side chain analogues was available for valuating if the complicated pattern of metabolism noted by Brown

and Boyd (2) was due to the absence of an intact side chain.

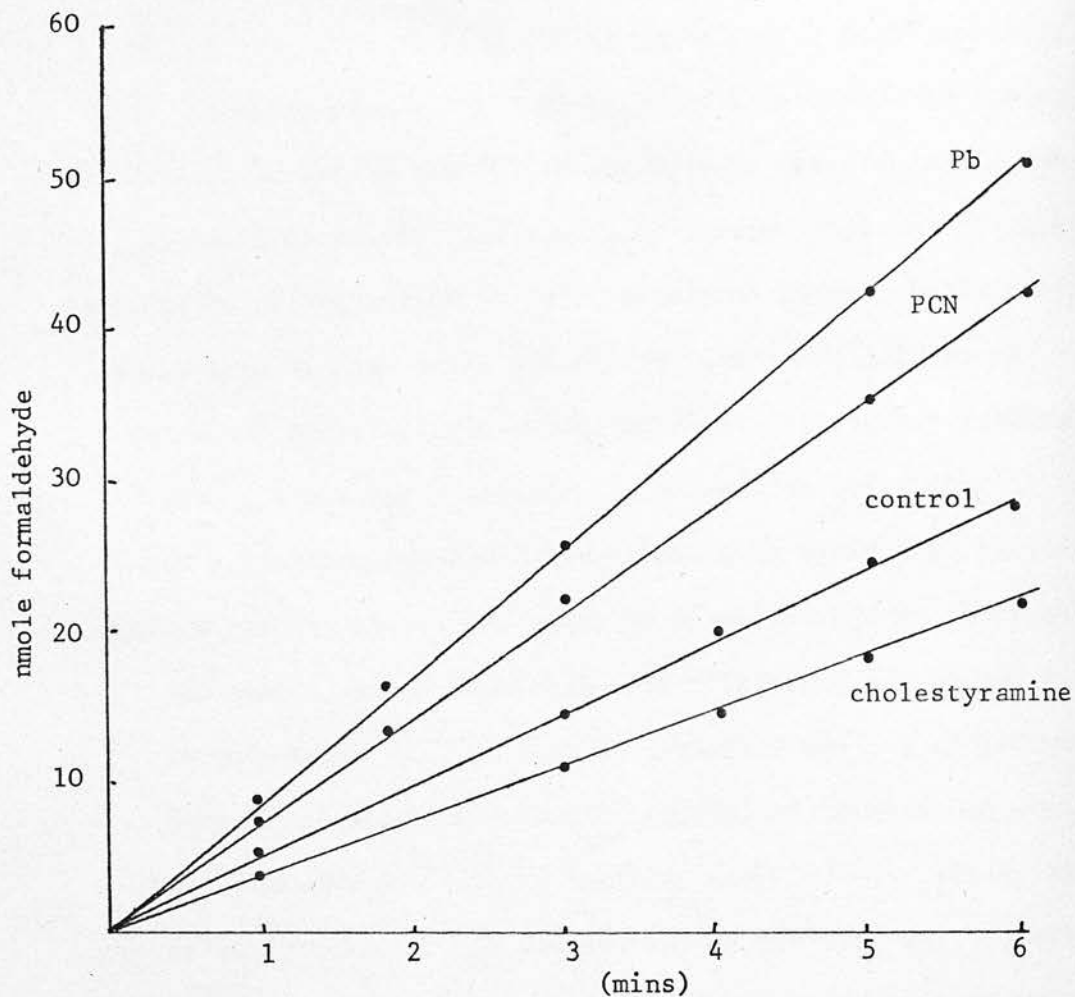
In fact the results of the study showed that cholesterol side chain analogues were hydroxylated by the liver in a manner which is determined by their side chain structure, 26-nor-cholesterol ( $C_{26}$ ), (Fig. 2.23) was hydroxylated to a  $7\alpha$ -hydroxy derivative to an extent which is 65% that of cholesterol, 26-dimethyl-27-norcholesterol ( $C_{28}$ ) formed a single product, under the same assay conditions, at 10% the extent of cholesterol hydroxylation and cholenol ( $C_{24}$ ) was metabolised to a single unidentified product consistent with  $7\alpha$ -hydroxylation of cholenol.

Shortening the sterol side chain still further appeared to introduce the possibility of a completely different mechanism for the oxidative metabolism of sterols.  $C_{21}$  and  $C_{22}$  were oxidised to a range of products which was not consistent with a specific  $7\alpha$ -hydroxylation of these shorter side chain analogues. This led to the proposal that a different enzyme system was responsible for the oxidation of short side chain analogues in the endoplasmic reticulum. Boyd et al. (10) postulated that the  $C_{21}$  and  $C_{22}$  were oxidised by the drug metabolising system. The activity of the cholesterol  $7\alpha$ -hydroxylase enzyme was unaffected by pretreatment with phenobarbitone at least in the case of Wistar strain rats (121). The effects of drug mediated induction on enzymes of the endoplasmic reticulum have been well documented (122).

The pretreatments used in this study to try to elucidate the metabolic fate of  $C_{21}$  and  $C_{22}$  involved the use of a selection of recognised drug inducing agents, (a) phenobarbitone (Pb), (b) the polycyclic hydrocarbon 3-methyl-cholanthrene (3MC), and (c) pregnenolone-16 $\alpha$ -carbonitrile (PCN). Many compounds



Fig. 6.1(a)



Treatment	n mole cytochrome P450/mg protein	n mole cytochrome b <sub>5</sub> /mg protein
control	0.385	0.23
cholestyramine	0.302	0.24
Pb	0.714	0.29
P.C.N.	0.6	0.26

Fig. 6.1(b)

Fig. 6.1(a)

The effect of Pb, PCN, and cholestyramine on ethylmorphine-N-demethylase from ♂ rat liver microsomes.

Fig. 6.1 (b)

Comparison of the effect of Pb, PCN, and cholestyramine on the levels of two components of the ethylmorphine-N-demethylase found in rat liver microsomes.

behave like phenobarbitone in stimulating varied pathways of xenobiotic metabolism by liver microsomes including oxidation and reduction reactions, glucuronide formation and de-esterification. In contrast polycyclic aromatic hydrocarbons stimulate a more limited group of reactions. The differences between Pb and 3MC inductions have helped in the identification of several different hydroxylating enzyme systems in rat liver. N-demethylation of ethylmorphine by liver microsomes from normal or Pb treated rats was inhibited by SKF 525A whereas that from rats treated with 3MC was not (123). In addition Pb administration increased the maximal velocity of ethylmorphine-N-demethylation without influencing the Michaelis constants (124).

The three types of inducers differ in course and intensity of induction

- (a) on daily administration of phenobarbitone to rats by intraperitoneal injection or by inclusion in drinking water, the maximal increase in enzyme activity (3-10 fold) is not reached for at least 3 days.
- (b) the induction with 3MC reached its maximum stimulation (5-10 fold) 24 hours after i.p. injection.
- (c) the stimulation induced by PCN required delivery by gastric incubation in Tween:water and 3 doses given at 24 hour intervals (122). The enzyme activity is stimulated 2-5 fold within 3 days.

In addition the mechanism by which the stimulation is achieved is slightly different in each case. Although both Pb and 3MC increase P450 (P448 in the case of 3MC), NADPH cyt c reductase and perhaps cytochrome  $b_5$  levels (125) Pb administration causes a marked proliferation of smooth surfaced membranes of the

endoplasmic reticulum while 3MC has no effect.

Using inbred strains of mice Haugen et al. (126) showed that the Pb mediated stimulation was as a result of de novo synthesis of cytochromes. Four polypeptides were detected which corresponded to species formed by drug inducements; Pb, PCN and 3MC induced polypeptides with MW 51000, 54,000 and 55,000 respectively. In addition, Pb induced a 49,000 MW polypeptide but to a lesser extent than the 51,000 MW species.

Thus using these proven inducing agents of the drug metabolising systems it should be possible to further investigate the differences that alteration of the cholesterol side chain confers on hepatic sterol metabolism.

## 6.2 The effect of drug administration on endoplasmic reticulum

As a means of determining if the administered compounds were causing an alteration in the activity of the drug metabolising enzymes, the levels of cytochromes P450 and  $b_5$  and the activity of N-ethyl-morphine demethylase were determined in livers of male rats after treatment with Pb, cholestyramine and PCN. Figure 6.1 (a and b) shows that drug administration increased the specific content of cytochrome P450 without increasing cytochrome  $b_5$  and stimulated ethylmorphine-N-demethylase activity. In contrast the bile acid sequestrant cholestyramine had no effect in the amount of cytochrome P450 or  $b_5$  present and the ethylmorphine demethylase activity remained unaltered. These results show that a fully induced drug metabolising system was obtained.

The metabolism of  $[4-^{14}C]$ -cholesterol  $[^3H]$ - $C_{21}$  and  $[^3H]$ - $C_{22}$  was checked by adding acetone solutions of each sterol to a  $7\alpha$ -hydroxylase incubation medium containing NADPH generator and 10 mg of microsomal protein from control or treated rats. After an hour

Treatment	Sterol	%age of radioactivity in numbered fraction		Total %age of product	%age of radioactivity remaining in substrate.
		1-4	7-8		
control	C <sub>21</sub>	32	12	44	16.7
Pb.	C <sub>21</sub>	53	7	60	15
cholestyramine	C <sub>21</sub>	27	11	38	17
P.C.N.	C <sub>21</sub>	71	/	71	22
control	C <sub>22</sub>	14	12.2	26	60
Pb.	C <sub>22</sub>	31	15	46	30
cholestyramine	C <sub>22</sub>	15	5	20	57
P.C.N.	C <sub>22</sub>	44	/	44	50
control	C <sub>27</sub>	/	6.25	6.25	44
Pb.	C <sub>27</sub>	/	5.3	5.3	83
cholestyramine	C <sub>27</sub>	/	12	12	74
P.C.N.	C <sub>27</sub>	/	7	7	85

Comparison of the effect of Pb., P.C.N. and cholestyramine in the metabolism of C<sub>21</sub>, C<sub>22</sub> and C<sub>27</sub> by liver microsomes. Each value is the mean of at least 4 determinations.

Table 6.1

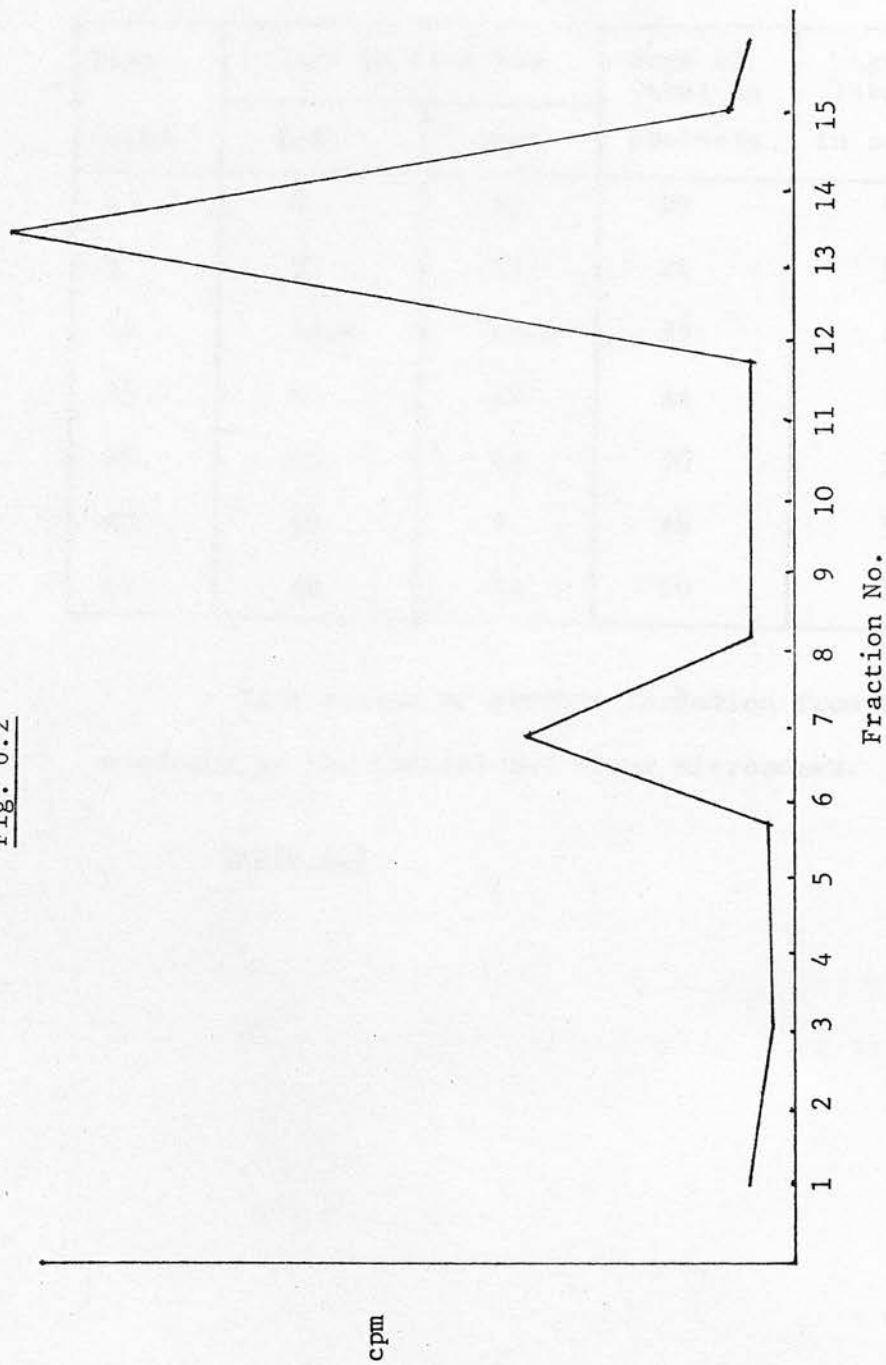
Time (min)	%age in fraction		%age of label in products	%age of label left in substrate
	1-4	rest		
2	8	13	21	61
5	9	17	26	52
10	14.4	18.6	33	47
15	26	18	44	28
20	25	14	39	35
40	40	8	48	31
60	48	12	60	23

Time course of product formation from  $C_{21}$   
analogue by Pb. induced rat liver microsomes.

Table 6.2

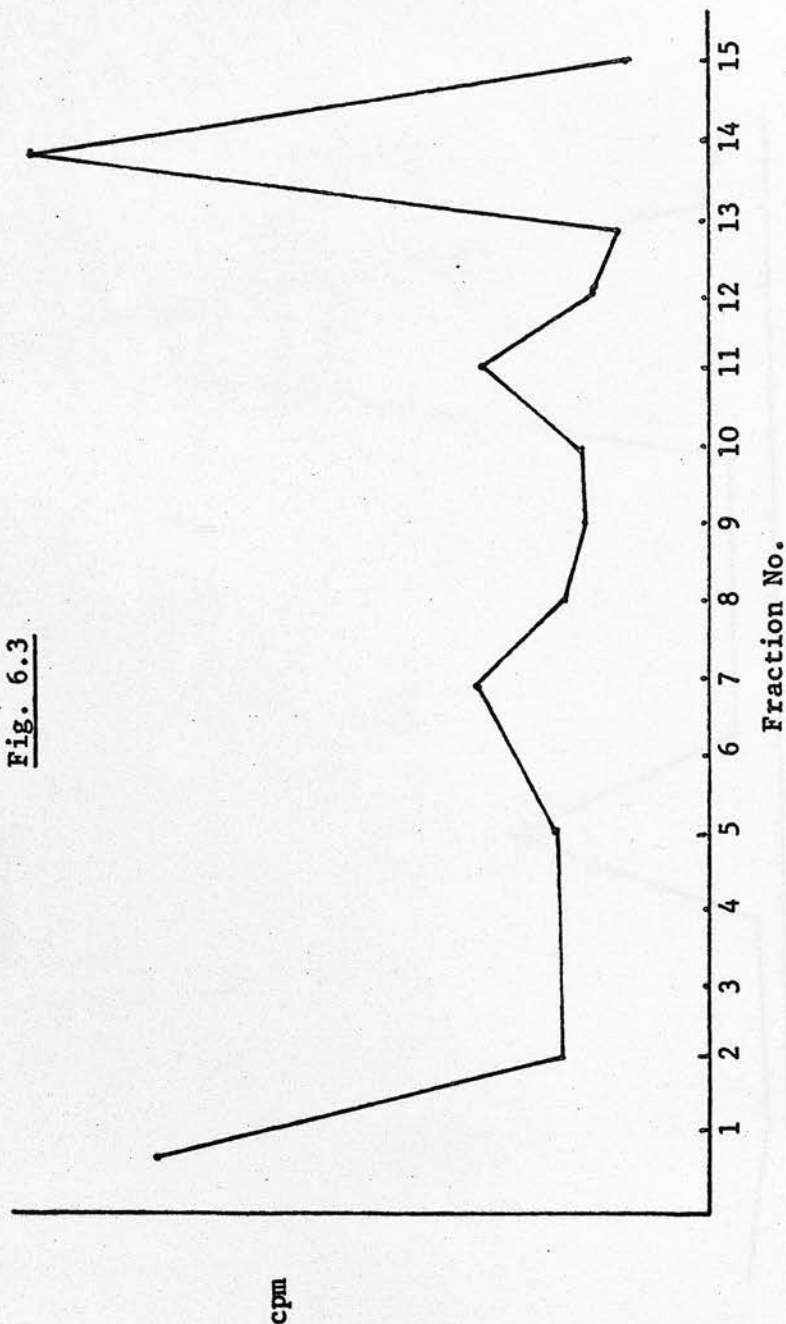


Fig. 6.2



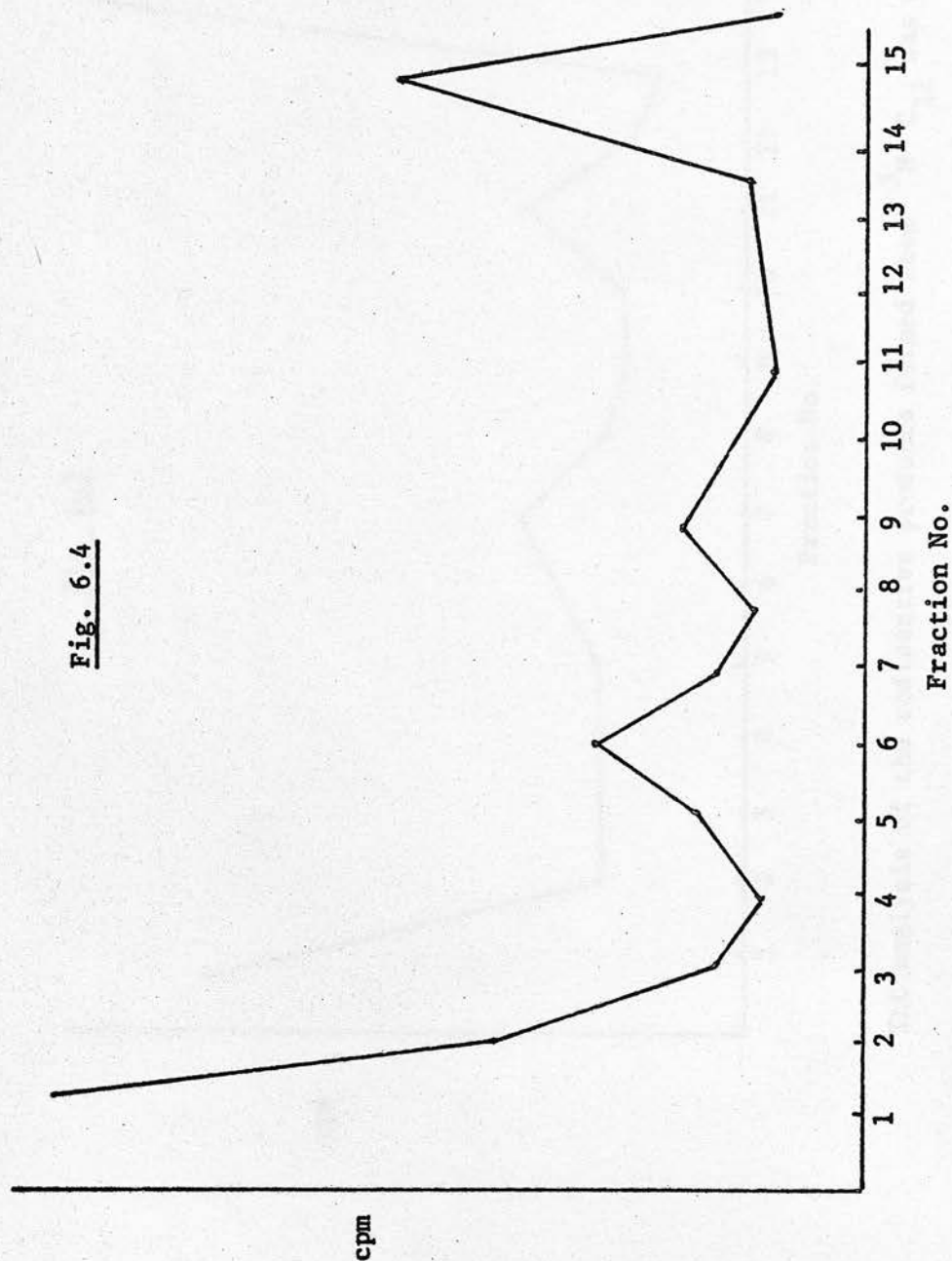
TLC analysis of the products formed when  $4\text{-}^{14}\text{C}$  cholesterol was metabolised by the microsomes in the presence of NADPH and cysteamine.

Fig. 6.3



TLC analysis of the radioactive products formed when  $^3\text{H}$   $\text{C}_{22}$  was metabolised by a microsomal preparation containing NADPH generator and  $\beta$  mercaptoethylamine.

Fig. 6.4



TLC analysis of the products formed when  $^3\text{H}$   $\text{C}_{21}$  was metabolised by a microsomal suspension.

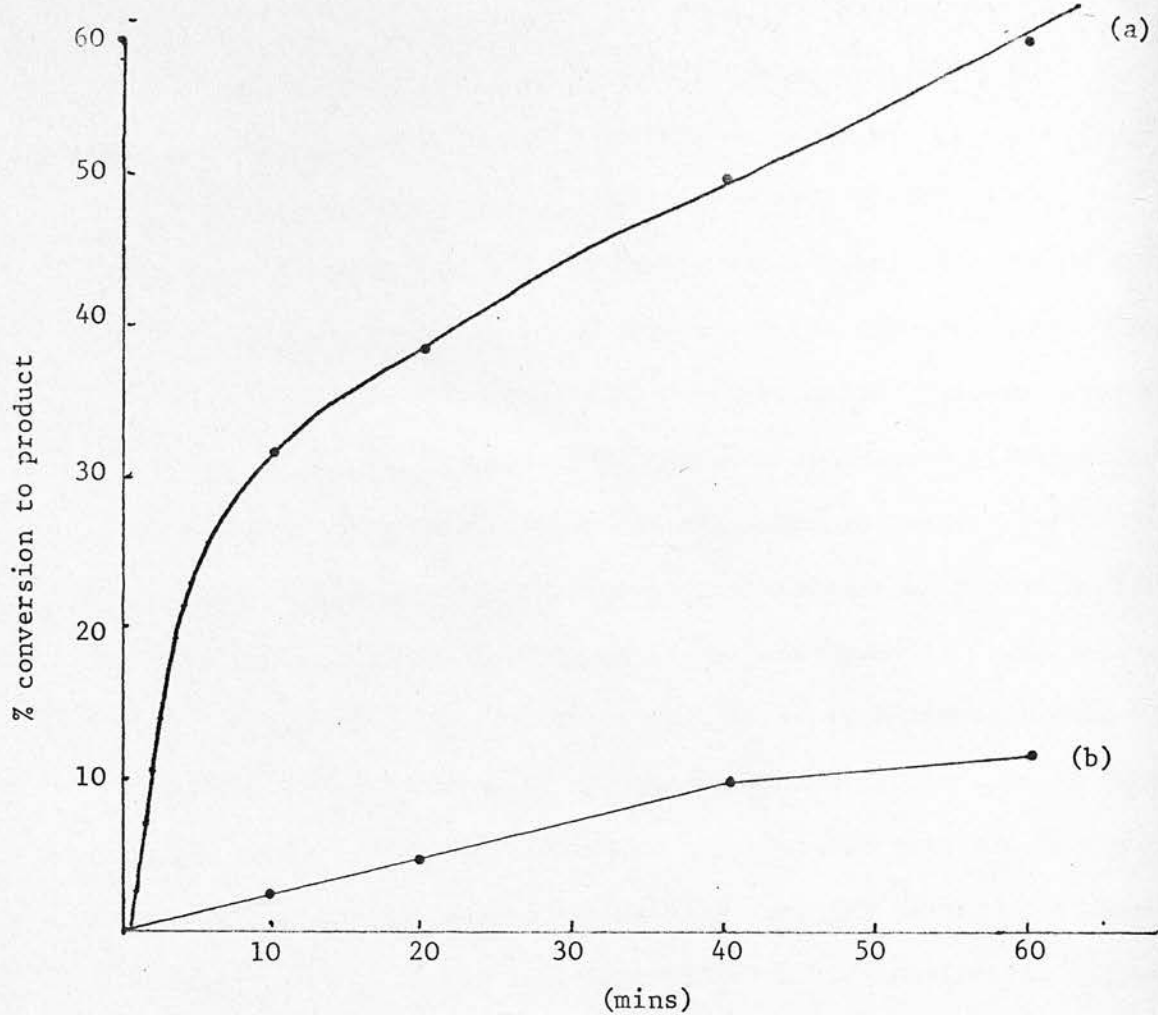
at 37°C the reactions were stopped and the lipids extracted.

Figure 6.2, 6.3 and 6.4 show the TLC scans of extracted hour incubations.

Table 6.2 shows that after a 2 minute incubation of  $[^3\text{H}]\text{-C}_{21}$  with 10 mg of Pb induced microsomal protein in the presence of NADPH generator, the polar fraction (1-4) only contained 8% of the recovered radioactivity. The proportion of the total radioactivity present in fraction (1-4) increased with increasing incubation time, whereas the radioactivity in other product fractions remained relatively constant throughout the incubation. Although the nature of the main polar product has not been determined it is perhaps a polyhydroxylated product which is only slowly formed due to its inability to gain immediate access to the drug metabolising enzymes. Figure 6.5 gives a good comparison between the time courses of cholesterol 7 $\alpha$ -hydroxylation and  $\text{C}_{21}$  metabolism. The differences observed in the rate and extent of cholesterol and  $\text{C}_{21}$  metabolism under identical assay conditions suggest that  $\text{C}_{21}$  and cholesterol are metabolised by different enzymes.

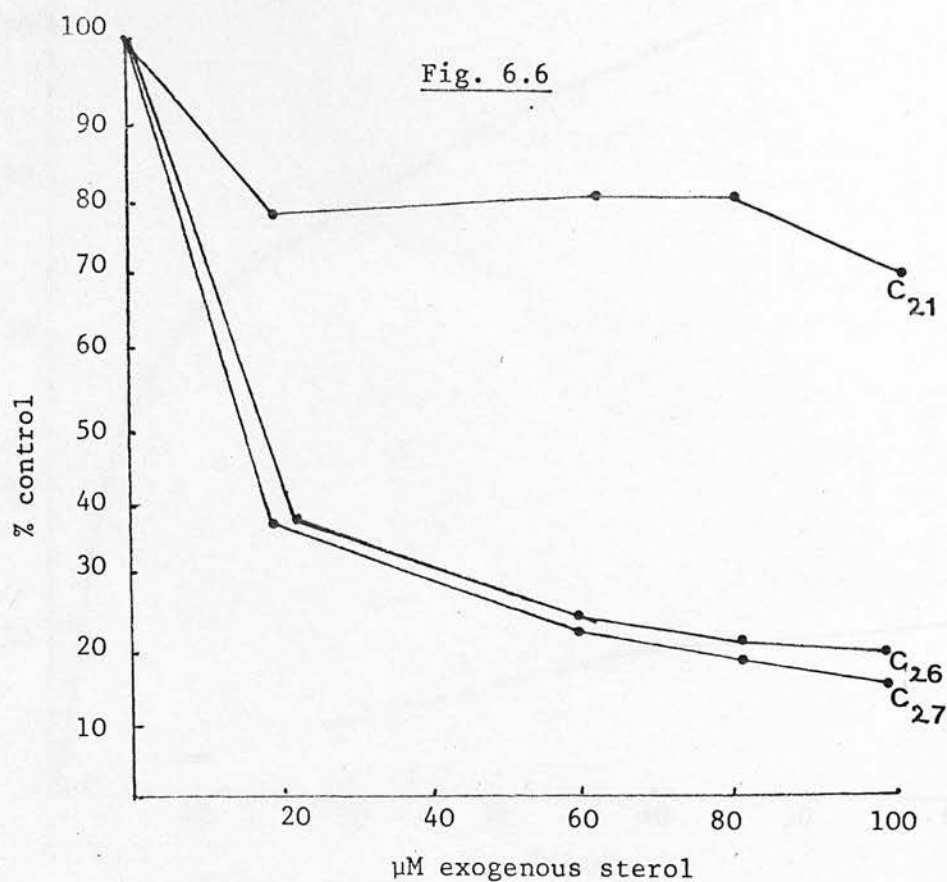
#### 6.4 The effect of the addition of a large excess of unlabelled sterol on cholesterol 7 $\alpha$ -hydroxylase activity

From the foregoing studies it is clear that alteration of the side chain of cholesterol resulted in observed differences in the metabolic fate of these cholesterol analogues. As the  $\text{C}_{24}\text{-C}_{28}$  analogues appeared to be metabolised in a similar manner to cholesterol (10) it was decided to investigate the effect of the addition of these previously studied steroids on the rate of 7 $\alpha$ -hydroxylation of cholesterol. In order to ensure that any effect noted was due to the presence of the exogenous analogue alone the experiments were carried out in intact microsomes and in a butanol extracted, Nonidet



Comparison between the time courses for (a) total metabolism of  $[^3\text{H}]\text{C}_{21}$  by Pb induced microsomes and (b) metabolism of  $[4\text{-}^{14}\text{C}]\text{cholesterol}$  by cholestyramine treated microsomes.

Fig. 6.5



sterol	% control value
C <sub>19</sub>	90
C <sub>21</sub>	88
C <sub>24</sub>	67
C <sub>25</sub>	62
C <sub>26</sub>	47
C <sub>27</sub>	31
C <sub>28</sub>	91
C <sub>29</sub>	83

Table 6.3



Fig. 6.6

The effect of the addition of varying amounts of cholesterol analogues on the production of 7 $\alpha$  hydroxycholesterol by rat liver microsomes.

Table 6.3

The effect of the addition of 100  $\mu$ M unlabelled cholesterol analogues on the production of 7 $\alpha$  hydroxycholesterol by a Nonidet solubilised, butanol powder of rat liver microsomes.

P42 solubilised, microsomal preparation. The butanol removes endogenous cholesterol which could interfere with the transport of the analogue to the enzyme active site. The experimental design in both cases was identical and involved the addition of up to 100  $\mu\text{M}$  exogenous analogue to a standard 7 ml incubation mixture containing 10 mg protein and radioactive cholesterol. The effect of the unlabelled exogenous cholesterol analogue was quantified by expressing the amount of  $7\alpha$ -hydroxycholesterol formed in the presence of the cholesterol analogue as a percentage of the control value. Figure 6.6 shows the effect of 3 added analogues on cholesterol  $7\alpha$ -hydroxylase of rat liver microsomes. The results show that even in the presence of 200  $\mu\text{g}$  of endogenous cholesterol,  $\text{C}_{26}$  and  $\text{C}_{27}$  cause a much greater reduction of  $7\alpha$ -hydroxycholesterol formation than does  $\text{C}_{21}$ . When part of the experiments were repeated with a Nonidet solubilised butanol extracted preparation and a fuller range of non-radioactive analogues at 100  $\mu\text{M}$  (Table 6.3) a similar result was obtained. This result confirmed the previous findings that cholesterol  $7\alpha$ -hydroxylase has a specific substrate requirement for a sterol molecule with an iso-octane side chain.

#### 6.5 An absolute assay for cholesterol $7\alpha$ -hydroxylase

The use of a more precise assay could improve experiments outlined in the previous sections that were designed to further illustrate the side chain dependence of cholesterol  $7\alpha$ -hydroxylase. The cholesterol analogue that was added to the incubation mixture with radioactive cholesterol was present in a vast excess over the radioactive cholesterol. The results obtained were accordingly limited in their application and difficult to interpret in mechanistic terms, because the radioactive cholesterol used to monitor the enzyme

activity constituted such a small part of the total sterol pool. In addition the radioactive cholesterol assay involved the assumption that the tracer amounts of cholesterol added achieved complete equilibration with all the cholesterol of the endoplasmic reticulum and that the  $7\alpha$ -hydroxycholesterol formed had the same specific radioactivity as total cholesterol. The extent of the equilibrium with all the methods is also thought to be determined by the form that the exogenous cholesterol is presented to the enzyme. When cholesterol is presented to the enzyme in an emulsion the rate at which the added cholesterol is  $7\alpha$ -hydroxylated may be influenced by the particle size (127). Hence apparent differences in enzyme activity may be due to the physical state of the exogenous substrate rather than to differences in the capacity or degree of activation of the enzyme. The most common methods of addition of cholesterol are in an organic solvent (10), or in a solution of a non-ionic detergent (11). However in intact microsomes the presence of a large excess of endogenous cholesterol complicates the interpretation of results and one cannot be sure that the behaviour of an enzyme <sup>o</sup>towards exogenous cholesterol is identical with its behaviour towards endogenous cholesterol.

A cholesterol  $7\alpha$ -hydroxylase assay system involving the addition of a cholesterol molecule tritiated at the  $7\alpha$  position to the microsomal enzyme system has been developed by Van Cantfort et Gielen (128). The assay was based on the assumption that  $7\alpha$  hydroxylation of this specifically labelled substrate will result in the release of  $^3\text{H}_2\text{O}$  which can be detected by liquid scintillation. Although this method is quicker and does not involve lipid extraction or thin layer chromatography of mixtures to isolate the desired <sup>product</sup> it does suffer from two major disadvantages (a) the  $[7\alpha\text{-}^3\text{H}]$ -cholesterol is

difficult to synthesise (b) the equilibration problems described for radiotracer assay also apply in this case.

In order to overcome these disadvantages a number of workers have developed cholesterol 7 $\alpha$ -hydroxylase assays which determine the actual mass of 7 $\alpha$ -hydroxycholesterol formed during the assay. Mitropoulos and Balasubramaniam (129) added a trace of 4-<sup>14</sup>C - cholesterol to the microsomal incubation mixture as an exogenous substrate. The radioactive 7 $\alpha$ -hydroxycholesterol present at the end of the incubation was then isolated from the total sterol fraction by TLC and acetylated with tritiated acetic anhydride of known specific radioactivity. The doubly labelled diacetate of 7 $\alpha$ -hydroxycholesterol was purified and its <sup>3</sup>H and <sup>14</sup>C radioactivities determined. The mass of 7 $\alpha$ -hydroxycholesterol recovered as the diacetate could then be calculated from the known specific radioactivity of the tritiated acetyl group, measurement of the <sup>3</sup>H/<sup>14</sup>C ratio also making it possible to correct for losses of sample. The mass of 7 $\alpha$ -hydroxycholesterol formed during the incubation was estimated from the difference between the amount present at the beginning and end of the incubation. This is a rather complex procedure.

Another means of measurement of the mass of 7 $\alpha$ -hydroxycholesterol has been developed using gas liquid chromatography alone or gas liquid chromatography combined with mass spectrometry. Malinow et al. (80) determined the mass of 7 $\alpha$ -hydroxycholesterol present after an incubation, thin layer chromatography and extraction of the silica gel with methanol. Trimethyl silyl (TMS) ethers of 7 $\alpha$ -hydroxycholesterol were prepared and injected on to a 1% SE30

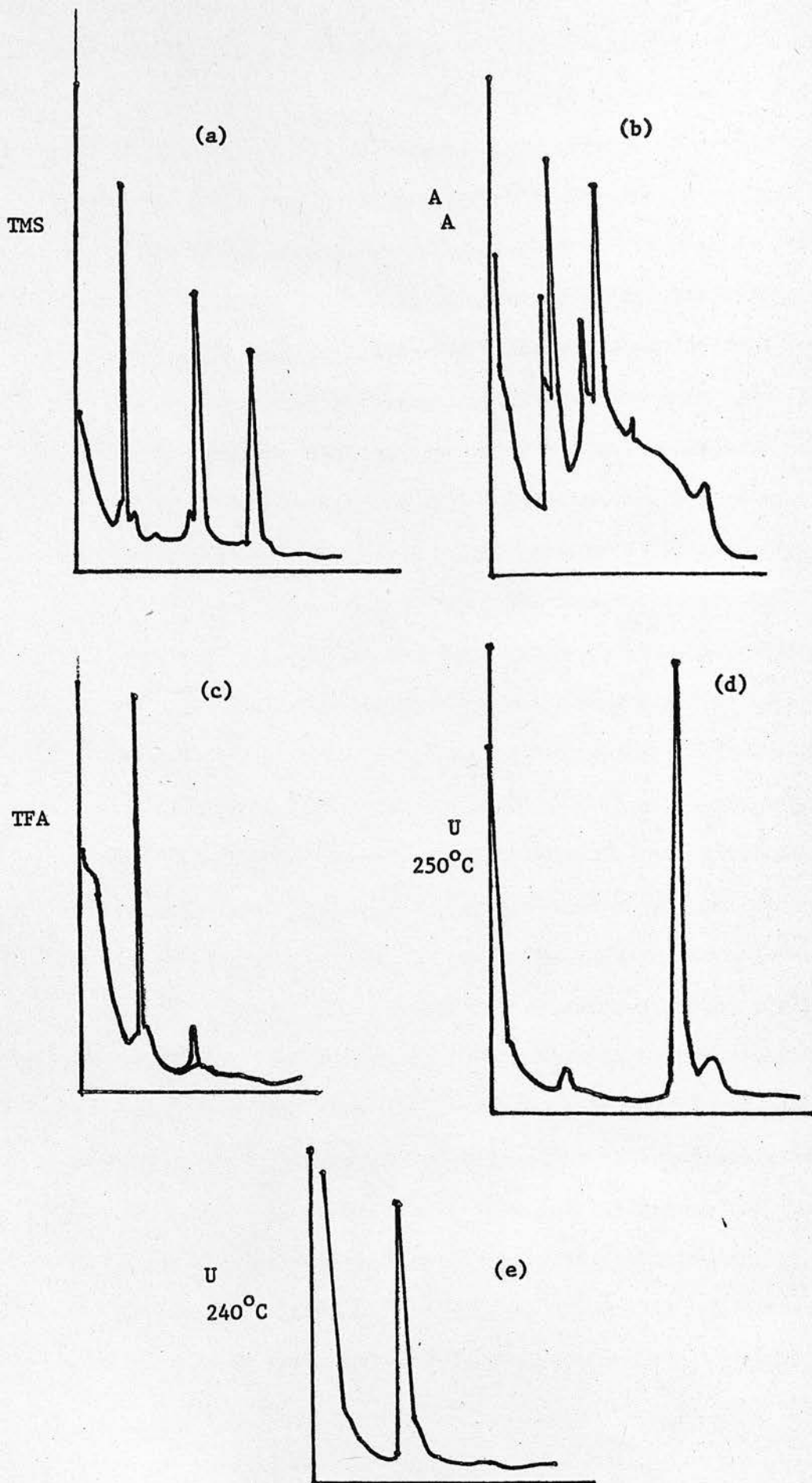


Fig. 6.7



Fig. 6.7

Sample traces obtained when 7 $\alpha$  hydroxycholesterol is injected onto a 1% SE30 GLC column.

- (a) Analysis of the TMS derivative.
- (b) Analysis of the diacetate derivative.
- (c) Analysis of the di-trifluoroacetate derivative.
- (d) Analysis of the underivatized material with a column temperature of 205°C.
- (e) As (d) but with a column temperature of 240°C.



on 100/120 mesh gas chrom Q column maintained at 240°C with the detector temperature at 240°C, the injection port temperature 250°C and a nitrogen flow of 35 cm per minute. The mass of 7 $\alpha$ -hydroxycholesterol formed was determined after taking into account losses in the radioactivities of internal standards.

This method has been refined by the use of a mass spectrometer attached to the outlet of a GLC column. After the termination of incubation, Bjorkhem and Danielsson (11) added a known amount of trideuterated 7 $\alpha$ -hydroxycholesterol. A chloroform methanol extract of the incubation mixture was subjected to TLC and the fraction containing 7 $\alpha$ -hydroxycholesterol was converted into the TMS ether and subjected to combined GLC-mass spectrometry. The ratio between the relative intensities of the nature peaks at m/e 456 corresponding to the native and m/e 459 corresponding to the deuterated allowed determination of the 7 $\alpha$ -hydroxycholesterol formed from endogenous cholesterol after taking into consideration the extent of dilution of the deuterated molecules with undeuterated. This method entails the preparation of deuterated 7 $\alpha$ -hydroxycholesterol and TLC purification of the extract.

A new mass spectrometric procedure has recently been published which avoided the use of a specifically labelled substrate or recovery marker, or TLC (12). This method involves the injection of TMS ethers present in a chloroform-methanol extract of an incubation directly onto a 2 m by 3 mm glass column packed with 1% SE30 on 100-120 mesh gas chrom Q. The TMS sterol ethers present are detected by multiple selected ion monitoring and the endogenous cholesterol present serves as an internal standard. Although this method is by far the easiest and quickest assay described in the

literature it requires the use of a highly sophisticated, expensive piece of apparatus. In the following six sections the development of a GLC assay for  $7\alpha$ -hydroxycholesterol and its subsequent use for preliminary investigation of methods of supplying substrate to the cholesterol  $7\alpha$ -hydroxylase enzyme will be described.

#### 6.6 Development of a gas liquid chromatographic assay for cholesterol $7\alpha$ -hydroxylase

Liver microsomes contain approximately 500 nmols of cholesterol per gram wet weight. This cholesterol pool is not necessarily homogeneous as the cholesterol is continually being synthesised and degraded and augmented by intestinal absorption. The disadvantages associated with the radioactive cholesterol assays have already been mentioned and thus it was decided to try to improve the existing mass assays involving the use of gas liquid chromatography.

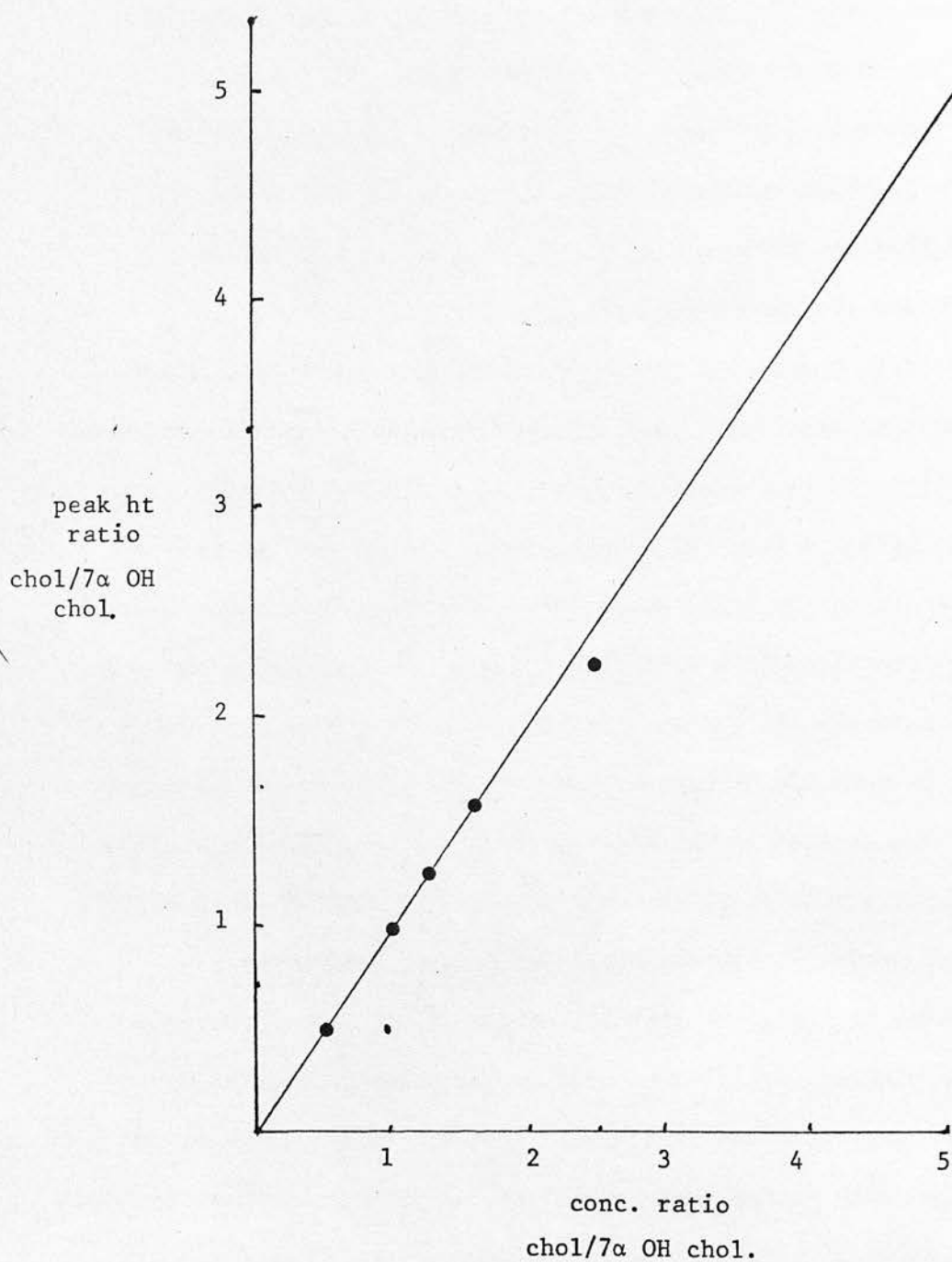
The initial attempts at the development of a GLC assay for cholesterol  $7\alpha$ -hydroxylase followed the Malinow procedure (80). This method involved the preparation of a trimethylsilane derivative of  $7\alpha$ -hydroxycholesterol using 10 parts pyridine, 3 parts hexamethyldisilazone, and 1 part trimethyl chlorosilane as silylating reagent. The derivative so formed was applied to the 1% SE30 column with the column temperature set at  $240^{\circ}\text{C}$ . Analysis of the trace showed that three products were present (Fig. 6.7a) none of which could be attributed to the presence of unreacted TMS reagent. As the formation of the multiple products irrespective of the cause was undesirable another method of sample preparation detailed in (130) was investigated. This procedure involved exposing the  $7\alpha$ -hydroxycholesterol to the vapour of a

mixture (2:1) of dry pyridine and acetic anhydride for 16 hours at room temperature. The sample was dried, the diacetate was extracted by chloroform which was subsequently removed by nitrogen and an acetone was injected on to the same 1% SE30 column which was maintained at the same conditions as described above. Figure 6.7(b) indicates that many components were present in the acetone solution and that the acetylation procedure was not ensuring that only one form of product was detected. None of the peaks were due to pyridine contamination. This result is in contrast to previous published studies by Van Lier and Smith (13) who showed that the diacetate of 7 $\alpha$ -hydroxycholesterol does not decompose under the condition described.

In order to check that the previous result was due to incomplete acetylation the experiments were repeated exactly apart from the method of preparation of the diacetate derivative. The acetylating reagent was prepared from dry distilled tetrahydrofuran and tri-fluoroacetic anhydride (2:1) and added to the standard 7 $\alpha$ -hydroxycholesterol contained in a stoppered bottle. After 15 minutes at room temperature the solvent was removed under nitrogen followed by an hour in a vacuum desiccator and then the extract was dissolved in acetone and applied to the GLC column maintained at 240°C. This procedure gave a much improved conversion to the di-trifluoroacetate and only two products were detected (a) a minor peak with a retention time of 7 minutes and (b) a major peak with a retention time of 4.5 minutes (Fig. 6.7c). Under these column conditions injection of underivatised cholesterol gave a single sharp peak and its inclusion with derivatised samples of 7 $\alpha$ -hydroxycholesterol allowed the construction of a calibration curve for the determination of 7 $\alpha$ -hydroxylation. Unfortunately the GLC traces still contained an extra peak with a retention time which was midway between that of cholesterol

Fig. 6.8

GLC calibration curve for determination of  $7\alpha$   
hydroxycholesterol..



and 7 $\alpha$ -hydroxycholesterol diacetate, whose precise nature could not be identified.

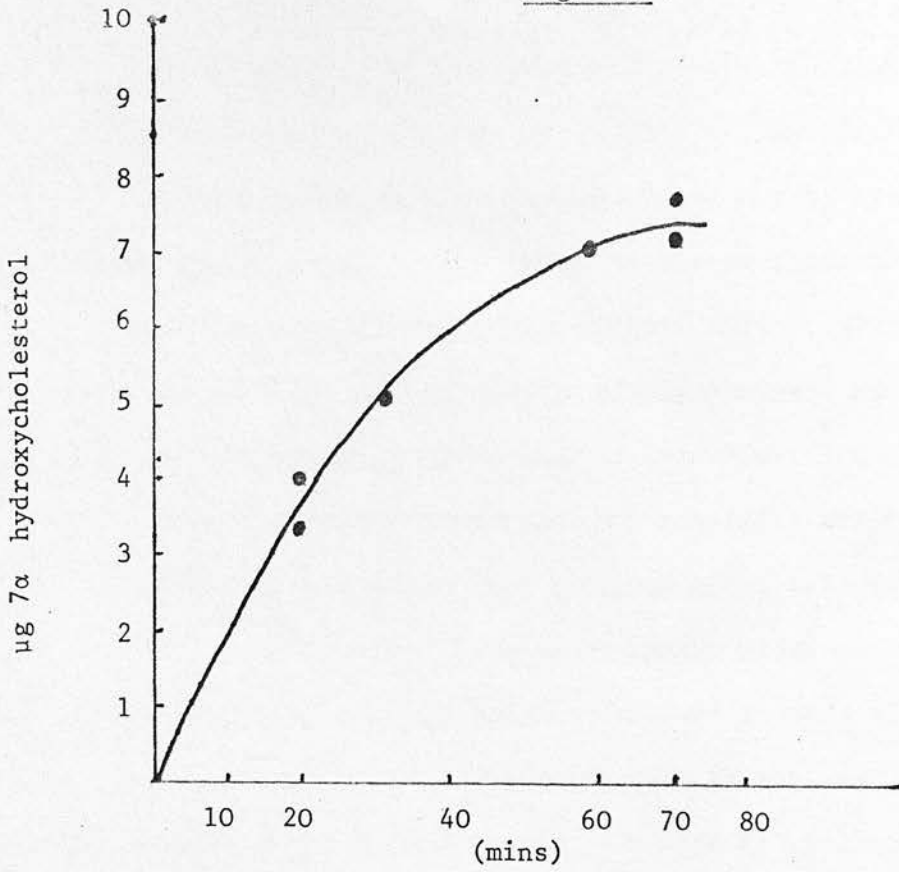
The method finally arrived at measured underivatized 7 $\alpha$ -hydroxycholesterol at a slightly lower column temperature (205°C). The full details of the assay procedure are described in the Materials and Methods section. Using this method a single peak was obtained (Fig. 6.7d). It should be mentioned that it is also possible to obtain a single product from 7 $\alpha$ -hydroxycholesterol at 240°C (Fig. 6.7e) which is in contradiction to the results of Van Lier and Smith (131) but the results of the experiments described in the following sections were determined at 205°C. This method is an improvement on the published procedure of Malinow (80) in that it does not require the preparation of a derivative. Thus the results obtained do not contain an error due to the inability to obtain a hundred % yield of derivative and the procedure employed contains fewer steps where recovery losses could occur.

#### 6.7 The behaviour of 7 $\alpha$ -hydroxycholesterol on gas liquid chromatography

Using the GLC conditions described in the Materials and Methods, underivatized 7 $\alpha$ -hydroxycholesterol gave a single symmetrical peak with a relative retention time as compared to cholesterol of  $0.55 \pm 0.4$ . This value is similar to that proposed by Brown (130) for the diacetate derivative (0.64) to the given value for the TMS derivative relative to cholesterol isobutyrate (0.57) (80). However these results are in contradiction to the results of Van Lier and Smith (131) who stated that a diacetate derivative of 7 $\alpha$ -hydroxycholesterol has a relative retention time compared to cholesterol of 1.69 on SE30 columns. The fact that the retention time of 7 $\alpha$ -hydroxycholesterol was less than cholesterol indicated that under the conditions used, injection of underivatized material

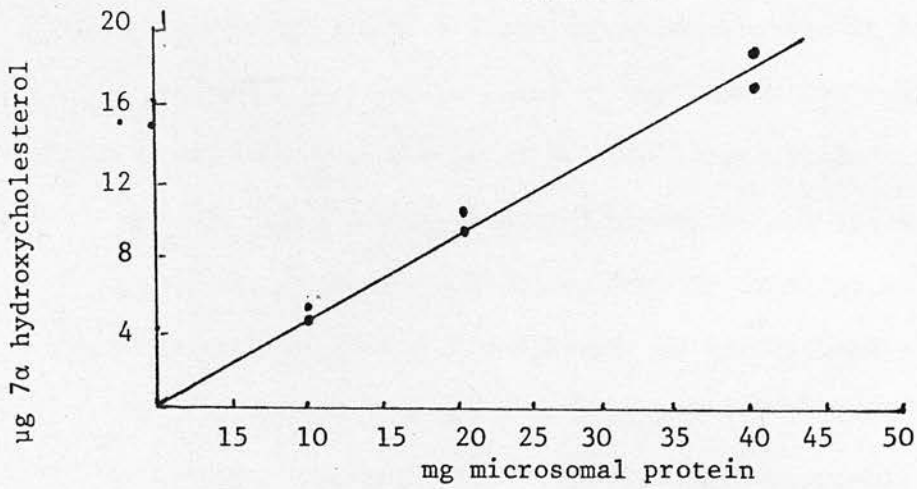


Fig. 6.9



Time course of production of 7α hydroxycholesterol by microsomes containing 9 mg protein.

Fig. 6.10



Graph showing dependence of 7α hydroxycholesterol production on protein.



resulted in a breakdown of the molecule into one product.

Assuming total decomposition of the  $7\alpha$ -hydroxycholesterol on injection it should be possible to obtain a quantitative measurement of the mass of  $7\alpha$ -hydroxycholesterol present in an incubation by constructing an assay based on the fact that a single symmetrical peak was obtained. However it is essential that a calibration curve with varying amounts of cholesterol and  $7\alpha$ -hydroxycholesterol be run with each set of assays. This was seen to be necessary as the gradient of the graph of peak height ratios versus concentration ratios added (calibration curve) varied between 1 and 2, although symmetrical peaks were obtained and similar column conditions were employed. A typical calibration curve is shown in Fig. 6.8.

This method is not sufficiently sensitive to discriminate between the  $7\alpha$  and  $7\beta$  isomers, but the preliminary TLC step will remove interfering  $7\beta$  isomers.

#### 6.8 The effect of the incubation period and the total amount of protein present on the production of $7\alpha$ -hydroxycholesterol

As an initial test of the assay procedure the relationship between product formation and the incubation time and the protein concentration was determined. Figure 6.9 shows the time course obtained when fresh microsomes containing 10 mg of protein and 150  $\mu$ g cholesterol were incubated for varying periods of time. The pattern of  $7\alpha$ -hydroxycholesterol production is similar to that obtained for the tracer assay and the decrease in the rate of product formation with increasing time conforms to the published data for this process (80). In addition the value for the amount of material produced per hour is on average 10-15 ng/min/mg protein which is comparable with published results for cholestyramine treated

protein (mg)	endogenous cholesterol ( $\mu$ g)	exogenous cholesterol ( $\mu$ g)	7 $\alpha$ hydroxy- cholesterol ( $\mu$ g)
20	300	0	9
20	300	50	9
20	300	100	8.6
20	300	150	9
20	300	200	10.1
20	300	250	8

Values for 7 $\alpha$ hydroxycholesterol  $\pm 0.5$  g  
and are the means of 4 determinations.

The effect of the addition of exogenous cholesterol  
in acetone on 7 $\alpha$ hydroxycholesterol production by rat liver  
microsomes.

Table 6.4

B.P. protein (mg)	exogenous cholesterol ( $\mu$ g)	7 $\alpha$ hydroxy- cholesterol ( $\mu$ g)
9	40	3.8
9	100	4.5
9	160	6.0
9	200	3.8
9	240	6.33
9	320	3.00
9	400	4.30

Results are mean  $\pm 0.5$  and are the results of  
4 determinations.

The effect of exogenous cholesterol (in acetone)  
on 7 $\alpha$ hydroxycholesterol production by a butanol powder of  
rat liver microsomes.

Table 6.5

rats. Malinow data expressed in the same units gave the value as 9.2 (80) which agreed with the previously published figure of Mitropoulos (132). Figure 6.10 shows that the production of 7 $\alpha$ -hydroxycholesterol is proportional to the amount of microsomal protein present.

#### 6.9 Investigation of the supply of cholesterol to the enzyme

In order to investigate if the rate of production of 7 $\alpha$ -hydroxycholesterol observed in the intact microsomes was limited by substrate supply, increasing amounts of cholesterol in acetone were added to fresh microsomes containing 150  $\mu$ g endogenous cholesterol and 10 mg of protein and the amount of 7 $\alpha$ -hydroxycholesterol produced by this system was measured by GLC. Table 6.4 shows that the addition in 50-400  $\mu$ g exogenous cholesterol in acetone (50  $\mu$ l) has no effect on the production of 7 $\alpha$ -hydroxycholesterol. When the exogenous cholesterol was added in Tween 80 a similar pattern to that obtained with acetone was observed. These results indicate that the addition of exogenous cholesterol to intact microsomes has no effect on the mass of 7 $\alpha$ -hydroxycholesterol produced by endogenous cholesterol. The question of the substrate saturability of the enzyme has been discussed in the literature. Initially Bjorkhem and Danielsson (133) reported that the addition of cholesterol in acetone to the incubation medium did not increase the yield of 7 $\alpha$ -hydroxycholesterol and thus they suggested that the endogenous substrate was saturating the enzyme under assay conditions. In a more recent paper (11) they compared the addition of exogenous cholesterol in acetone and in Tween 80 and noted that the degree of equilibration of exogenous cholesterol with endogenous cholesterol depended on the amount of cholesterol added and the physical state in which it was presented. Using

Tween 80 to introduce increasing amounts of exogenous cholesterol they could influence the production of  $7\alpha$  hydroxycholesterol, indicating that the  $7\alpha$ -hydroxycholesterol hydroxylase is not saturated with substrate under these conditions. They calculated that the substrate pool of cholesterol  $7\alpha$ -hydroxylase maximally corresponded to about one third of the total amount of cholesterol normally present in the microsomal fraction. This figure differs from the 70% quoted as available to the enzyme by Van Cantfort et al. (128). All the experiments that report an increase in formation of  $7\alpha$ -hydroxycholesterol on the addition of exogenous cholesterol utilised detergent solutions to deliver the substrate. In addition to providing an extra source of cholesterol the detergent disrupts the membrane structure, destroying the intermolecular organisation normally operative in the membrane. The question of enzyme saturation therefore remains a matter which cannot be definitely answered using either of these two techniques to supply substrate to enzyme.

#### 6.10 The influence of exogenous cholesterol on the $7\alpha$ -hydroxylase activity of a cholesterol depleted microsomal preparation

In an attempt to clarify the position regarding the supply of substrate cholesterol to the enzyme, a butanol extract of microsomes which retained the activity of the enzyme as judged by the oxidation of radioactive cholesterol, but did not contain any endogenous cholesterol, was prepared and treated with cholesterol in various physical forms. In all the experiments to be described the cholesterol was preincubated with the butanol powder for 1 hour at  $37^{\circ}\text{C}$  prior to the addition of the NADPH generating system. The microsomal extract was then incubated for a further hour and the mass of  $7\alpha$ -hydroxycholesterol formed was determined as previously described. The

Butanol Powder protein (mg)	exogenous cholesterol ( $\mu$ g)	bound cholesterol ( $\mu$ g)
10	40	35 $\pm$ 2
10	80	70 $\pm$ 2
10	120	73 $\pm$ 2
10	150	105 $\pm$ 4
10	160	130 $\pm$ 4
10	200	126 $\pm$ 4
10	240	154 $\pm$ 4

Binding of cholesterol added in acetone to a butanol powder of rat liver microsomes. Results are mean  $\pm$  standard error of the mean.

Table 6.6

Butanol Powder protein (mg)	exogenous cholesterol ( $\mu$ g)	E.Y.PC (mg)	7 $\alpha$ -hydroxy- cholesterol ( $\mu$ g)
9	100	10	5.44
9	100	5	5.5
9	100	2.5	3.6
9	100	1	3.4
9	100	0.1	3.3
9	100	/	7 $\pm$ 0.5
9	trace	/	7% conversion

Comparison of the ability of liposomal cholesterol to act as substrate for cholesterol 7 $\alpha$ -hydroxylase.

Table 6.7



effect of the addition of an increasing amount of cholesterol in acetone is shown in Table 6.5. The results indicate that exogenous cholesterol added in acetone can act as a source of substrate for a cholesterol depleted preparation of microsomes. The results also show that (a) the rate of production of  $7\alpha$ -hydroxycholesterol does not increase in direct proportion to the increasing amounts of exogenous cholesterol presented to the membranes and (b) although the microsomes have been treated with organic solvents the enzyme activity of a butanol powder containing an equivalent amount of protein and exogenous cholesterol is comparable with the enzyme activity of intact microsomes of the same composition. This also implies that the cholesterol removed by the butanol contains cholesterol which acts as substrate for the enzyme.

#### 6.11 Investigation of the ability of a cholesterol depleted powder to sequester cholesterol added in acetone

The possibility that the failure of large amounts of cholesterol to stimulate ~~cholesterol~~  $7\alpha$ -hydroxycholesterol production was simply due to the fact that the butanol powder could not bind elevated amounts of cholesterol, was investigated. To a butanol powder reconstituted in 0.1 M phosphate buffer pH 7.4, containing 10.0 mg of protein and essentially no cholesterol (<2  $\mu$ g chol) varying amounts of cholesterol in acetone were added. The mixtures of protein and cholesterol were incubated at  $37^{\circ}\text{C}$  for 1 hour, then the protein was sedimented by centrifugation of the mixture at 100,000 g for 1 hour. The pellet thus obtained was resuspended in 0.1 M phosphate buffer pH 7.4 and the process was repeated. The supernatant was once more poured off and 10  $\mu$ l of  $4^{14}\text{C}$  cholesterol (0.1  $\mu$ c) was added to the pellet as a recovery marker



and the lipids were then extracted with chloroform:methanol (2:1). The tracer was added after the centrifugations as preliminary experiments had shown that a portion of the radioactivity was located in the supernatant which is discarded. The cholesterol present in each lipid extract was then determined either by GLC which required a further TLC step to separate cholesterol from phospholipids or by an enzymatic assay using cholesterol oxidase. The results obtained with both methods were identical and Table 6.6 shows that in every case approximately 85% of the cholesterol was bound to the butanol powder up to a maximum cholesterol:protein ratio of 0.013 mg/mg which is the approximate ratio found in the intact microsomes. Comparison of the binding of exogenous cholesterol to the butanol powder with the enzymatic conversion of cholesterol to 7 $\alpha$ -hydroxycholesterol by a similar preparation does not show any correspondence between the amount bound and the amount converted to product. Thus these cholesterol depletion experiments show that the removed cholesterol can be replaced by addition of exogenous cholesterol but they do not give any indication of the extent of substrate saturation of the enzyme.

#### 6.12 The effect of the addition of exogenous cholesterol to a butanol powder in the form of a liposome

As the form in which the exogenous cholesterol is added has been to play an important role in determining the extent of its metabolism (11), the effect of the inclusion of cholesterol in a liposome was compared with the results obtained from an acetone solution of cholesterol. Table 6.7 shows that the incorporation of the cholesterol in a multibilayered liposome does not improve the metabolism of the cholesterol to 7 $\alpha$ -hydroxycholesterol. This

could be due to the fact that unsonicated liposomes which consist of many layers of lipid were used which prevented the cholesterol gaining access to the enzyme active site in the hour preincubation time used in the study. The table also shows that the results of the mass assay and the tracer assay are directly comparable in the system. The assay described in Section 6.7 was developed to try to better define the possible mechanisms involved in the supply of substrate cholesterol to the cholesterol 7 $\alpha$ -hydroxylase enzyme. The use of a butanol powder of microsomes is open to the same criticisms that are levelled at the detergent mediated cholesterol transfer to microsomes in that the treatment of microsomes with organic solvents removes protein and phospholipid in addition to 99% of the cholesterol.

6.13 The effect of alteration of the cholesterol side chain on the production of pregnenolone by the cholesterol side chain cleavage enzyme from rat adrenal

The cholesterol side chain cleavage enzyme occurs in the mitochondria prepared from the cortex of adrenal glands and in common with other mixed function oxidases requires molecular oxygen and NADPH as cofactors. The early studies of Saba et al. (134) established that the enzyme converted cholesterol to pregnenolone, with the simultaneous formation of isocaproic aldehyde which could be subsequently oxidised to isocaproic acid. This enzymic conversion of cholesterol to pregnenolone is thought to be the rate limiting step in steroidogenesis and involves two hydroxylations followed by an oxidative cleavage of a C-C bond. A selection of the proposed molecular mechanism<sup>s</sup> for the hydroxylation reactions are catalogued in the review of Simpson and Mason (135),

however Dr. R. Hume has recently provided convincing evidence (136) that the side chain cleavage enzyme firstly converts cholesterol to 22-hydroxycholesterol which is immediately transformed to 20, 22-dihydroxycholesterol. This dihydroxy-compound is cleaved between carbon 20 and 22 with the formation of pregnenolone.

The side chain cleavage enzyme system consisting of an electron transport chain formed from the iron sulphur protein, adrenodoxin, and adrenodoxin reductase, with a unique cytochrome P450 (P450<sub>scc</sub>) as the terminal electron acceptor, is situated on the mitochondrial cristae (Fig. 1.3). Simpson et al. (137) have postulated the existence of a cholesterol pool within mitochondria that is specifically associated with cytochrome P450<sub>scc</sub> and suggest that a rate limiting process for cholesterol side chain cleavage is the ease with which the remaining cholesterol within the mitochondrion can form an active substrate cleavage product with cytochrome P450<sub>scc</sub>.

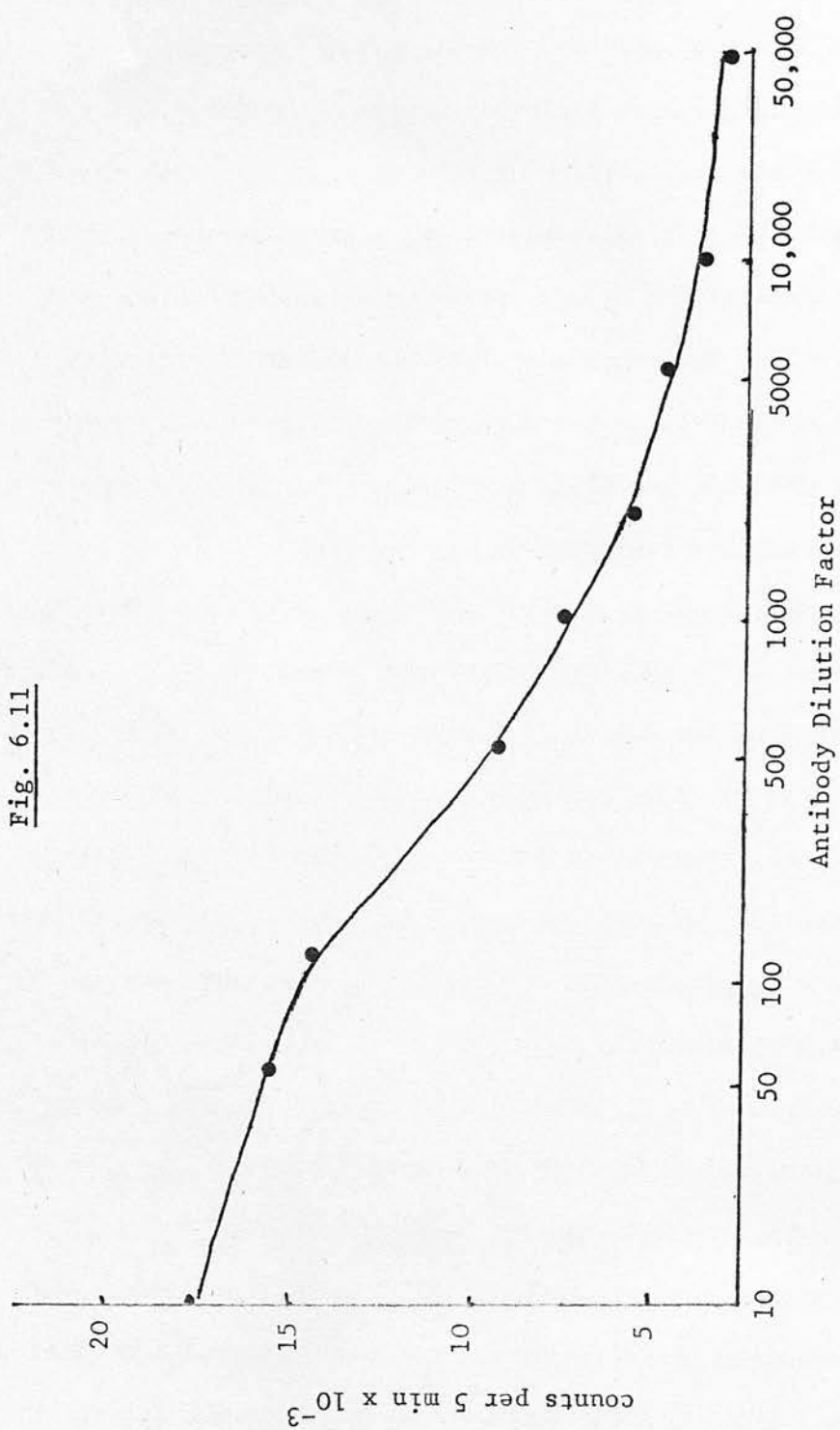
The activity of the side chain cleavage enzyme can be enhanced by treatment with adrenal cortex trophic hormone (ACTH). The mechanism of ACTH stimulation is not precisely understood. Koritz and Kumar (138) observed that adrenal mitochondria prepared from rats injected with ACTH showed an increased capacity for pregnenolone formation compared with adrenal mitochondria from control rats on incubating both sets of mitochondria in vitro with succinate. These results were interpreted in terms of the relative permeabilities of each mitochondrial preparation to pregnenolone. Thus ACTH pretreatment was thought to render the mitochondrial membrane very permeable to

pregnenolone, releasing pregnenolone mediated feedback inhibition of side chain cleavage. Based on their inability to observe any change in permeability of mitochondria to pregnenolone on ACTH treatment, Johnson et al. (139) suggested that pregnenolone feedback was not the most important regulating factor and surmised that the availability of substrate was most important. ACTH treatment of rats also reduced the mitochondrial stores of cholesterol in addition to its effect on cholesterol side chain cleavage (140). The ability of ACTH stimulated mitochondria to metabolise the cholesterol side chain analogues was studied in order to check if a similar specificity to that observed in the liver system was operative in the adrenal.

A previous report from this laboratory (141) has investigated the metabolism of these sterols by unstimulated adrenal mitochondria. The incubation medium used contained 250 mM sucrose, 20 mM KCl, 15 mM  $\text{NADP}^+$  and 0.6% BSA and cyanoketone. The reaction was initiated by the addition of D-L isocitrate (2.0 mM final conc) and the metabolism of each non-polar side chain analogue studied (10  $\mu\text{l}$  of a 4 mg/ml solution in ethanol) was similar to that obtained with cholesterol.

In the present work, adrenal mitochondria (0.5 mg protein) from ether stressed or ACTH pretreated rats were incubated with 10  $\mu\text{l}$  of 4 mg/ml cholesterol side chain analogue in an incubation medium containing sucrose (0.25 M), KCl (20 mM), triethanolamine (15 mM), potassium phosphate pH 7.4 (10 mM),  $\text{MgCl}_2$  (5 mM), 0.1% BSA and EDTA (0.1 mM). In addition each incubation contained 1  $\mu\text{mole}$  of calcium and 5 ml of cyanoketone (1 mg/ml) which prevents the pregnenolone from being further metabolised to progesterone. The side chain cleavage activity present was determined by assaying

Fig. 6.11



Pregnenolone Radio-Immuno-Assay Standard Curve

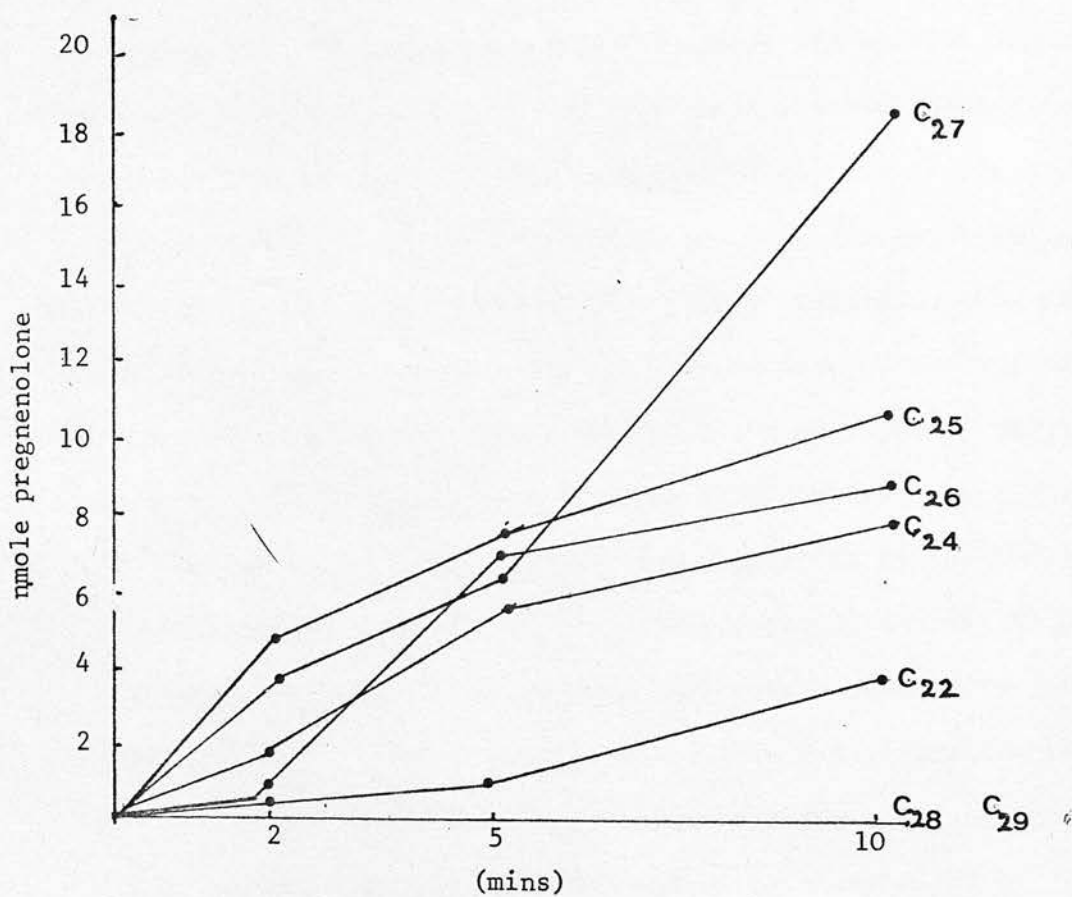


pregnenolone formation by means of a radioimmunoassay using anti-serum raised in New Zealand white rabbits against a pregnenolone-20-albumin conjugate.

Figure 6.11 shows the standard curve for the pregnenolone radioimmunoassay. Using the standard curve the amounts of pregnenolone formed from each cholesterol side chain analogue could be determined with mitochondria from ether stressed and ACTH treated rats. The results in Figures 6.12a and b are expressed relative to the pregnenolone produced by the mitochondria in the absence of exogenous sterol. The endogenous rate differs between ether stressed and ACTH pretreated mitochondria, being almost zero in ACTH treated animals which is consistent with the noted reduction in the cholesterol levels of ACTH treated adrenal mitochondria (140). The results show that the side chain cleavage enzyme when studied after a 3 minute preincubation of exogenous sterol in the presence of  $\text{Ca}^{2+}$  metabolises cholesterol optimally. It should be noted that  $\text{C}_{22}$ ,  $\text{C}_{28}$  and  $\text{C}_{29}$  are not metabolised to any extent and that  $\text{C}_{19}$  and  $\text{C}_{21}$  were not tested due to their structural similarities to pregnenolone and hence their ability to cross-react with the antibody. The results obtained are in contrast to the earlier study in our laboratory (141) which did not show side chain dependence.

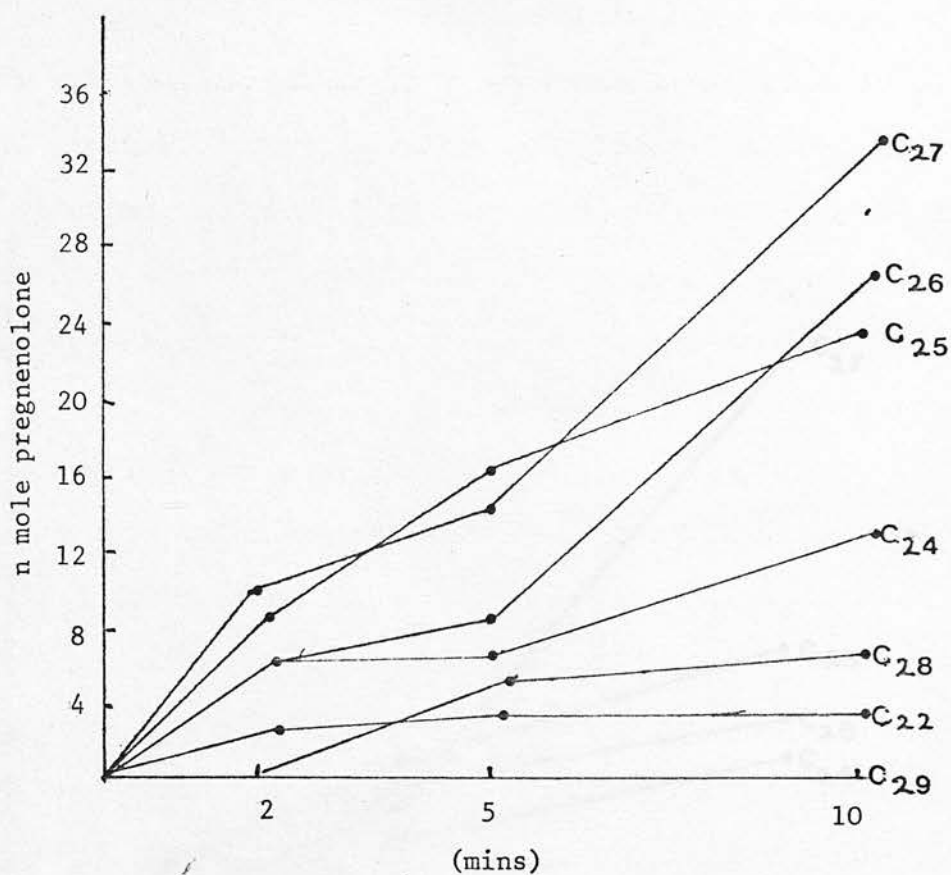
It is interesting to speculate why this side chain dependence was not detected in the untreated rats. In the previous study the metabolism of the exogenous sterols exhibited a biphasic pattern over 10 min incubation. However in the presence of  $\text{Ca}^{2+}$  (142) the initial rate of pregnenolone production is maintained over the assay period. The previously observed biphasic response was possibly due to the exhaustion of the available





Production of pregnenolone from cholesterol side chain analogues by adrenal mitochondria from ACTH pretreated rats.

Fig. 6.12 (a)



Production of pregnenolone from cholesterol side chain analogues by adrenal mitochondria from ether stressed rats.

Fig. 6.12(b)

steroidogenic pool of cholesterol in mitochondria. Perhaps  $\text{Ca}^{2+}$  acts to promote intramitochondrial cholesterol translocation to the side chain cleavage binding sites (143) by altering the lipid phase separation in the mitochondrial membrane.

The results in Figures 6.12a and b could be explained in terms of the relative affinity of each cholesterol side chain analogue for an ACTH stimulated enzyme, or their relative translational mobilities, through the mitochondrial membrane to the active site of the enzyme. Further study would be required to ascertain which mechanism was the more important or if a combination of both factors was determining pregnenolone production from each analogue.

### Conclusion

The results of both the cholesterol  $7\alpha$ -hydroxylase and the cholesterol side chain cleavage assays indicate that although the absolute values are seen to differ for different preparations (comparison of the ether stressed and ACTH treated rats (Fig. 6.12a and b)) the side chain of cholesterol precisely defines both the metabolic fate of the cholesterol molecule and the efficiency of its metabolism. This specificity noted for product formation may have contributions from the relative translation rates of the side chain analogue through the membrane. This may be related to the ability of cholesterol to interact with a phospholipid which enables it to gain rapid access to the enzyme. This would be consistent with the results of Chapters 4 and 5.

Another contribution to the observed metabolic fate of the various side chain analogues could be made by the relative interaction of the cholesterol analogues with the enzymes. This interpretation of the data would be consistent with some recent reports of cholesterol:protein interactions. Using a purified preparation

of band 3 protein from erythrocytes Klappauf et al. (14) demonstrated by monolayer studies that the protein would not bind phospholipid but gave a large interaction with cholesterol. On further investigation using a preparation of monolayers from  $C_{24}$  and  $C_{29}$ , they found that altering the side chain structure reduced this interaction — implying that the cholesterol side chain was determining the nature of the interaction between the cholesterol molecule and band 3 protein (144).

The nature of the side chain of a sterol has also been shown to be of importance in determining the specificity of cholesterol protein interaction observed in soluble proteins isolated from cell cytosol of mouse fibroblasts. By incubating  $[4^{14}C]$ -25-hydroxy-cholesterol and  $[3H]$  cholesterol with cell cytosol and subjecting the incubation to density gradient centrifugation, Kandutsch et al. (15) demonstrated that each sterol bound to a specific low molecular weight protein that aggregated in its presence. Thus the addition of a hydroxyl group at carbon 25 of cholesterol alters its protein binding properties in such a way that it cannot bind to the same protein as cholesterol.

These examples in combination with the experimental results described in this chapter indicate that the side chain dependence previously observed in cholesterol phospholipid systems also operates on the interaction between cholesterol and certain proteins. By ensuring that only cholesterol with its iso-octane side chain exhibits tight binding to specific proteins, the chances of other sterols especially plant sterols ergosterol and stigmasterol, interfering with cholesterol mediated cellular processes are almost negligible.

Another use of the cholesterol side chain analogues could be to investigate cholesterol protein interactions in a variety of membrane and cell cytosol systems.

CHAPTER 7

INVESTIGATION OF THE CHOLESTEROL POOL

FOUND IN MICROSCOPE

## 7.1 Introduction

In the previous chapter the problem of the supply of cholesterol to microsomes was investigated. That study used microsomes that had been depleted of cholesterol with butanol and attempts to remove endogenous cholesterol. In addition to removing cholesterol, butanol-acetone treatment removed about 25% of the phospholipids. As phospholipids are

## CHAPTER 7

cholesterol is important for optimal activation of the liver fatty  $\beta$ -hydroxylase enzyme (143) which will use

### INVESTIGATION OF THE CHOLESTEROL POOL

#### FOUND IN MICROSOMES

1450 (144), this may be in part due to the fact that the removal of cholesterol is necessary to increase the activity of a certain enzyme. Furthermore, it has been shown (145) that the lipid requirement for the activity of the enzyme is not a simple one but that it is a complex one. In the following chapter we will discuss the specificity of the cholesterol pool. Obviously, a method that removes cholesterol without affecting the rest of the microsomal components is to be preferred. In the present study we have used a method that removes cholesterol without affecting the rest of the microsomal components.

In this chapter the initial attempts to manipulate the amount of cholesterol in microsomes by treatment with liposomes are described. A related procedure can also be used to replace cholesterol by one of its analogues.

The treatment of microsomes with liposomes is described in this chapter. It is a technique that has been widely adapted to study the role of cholesterol in other cellular membranes. Principally, the levels of cholesterol in erythrocytes and ghosts (146) liposomes (147)



## Investigation of the Cholesterol Pool Found in Microsomes

### 7.1 Introduction

In the previous chapter the problem of the supply of cholesterol to cholesterol 7 $\alpha$ -hydroxylase was investigated. That study used microsomes that had been lyophilised and treated with butanol and acetone to remove endogenous cholesterol. In addition to removing cholesterol, butanol-acetone treatment removed about 25% of the phospholipids. As phosphatidylcholine has been shown to be essential for optimal activation of the liver fatty  $\omega$  hydroxylase enzyme (145) which like the cholesterol 7 $\alpha$ -hydroxylase enzyme is microsomal and cytochrome P450 linked, this removal of phospholipid may in part account for the inability of excess exogenous cholesterol to increase the enzyme activity of a butanol powder. Furthermore Lu and Coon (145) surmised that the lipid requirement for laurate hydroxylation implied that the lipid environment of the enzymes in the endoplasmic reticulum contributed to the substrate specificity. Obviously a method that removes cholesterol without affecting the rest of the microsomal components would be preferable to the butanol acetone treatment.

In this chapter the initial attempts to manipulate the amount of cholesterol in microsomes by treatment with liposomes are described. A related procedure can also be used to replace cholesterol by one of its side chain analogues.

The treatment of membranes with liposomes composed of phospholipid alone or a mixture of cholesterol and phospholipid is a technique that has been widely adapted to study the role of cholesterol in other cellular membranes. Principally the levels of cholesterol in erythrocytes and ghosts (146) lymphocytes (147)

and platelets (148) have been manipulated by the use of liposomes containing varying proportions of cholesterol or cholesterol analogues.

The erythrocyte has been the most popular membrane for these studies due to its ready availability and its high molar concentration of cholesterol in relation to phospholipid (1:1). It has been shown that cholesterol added in a liposome containing equimolar amounts of cholesterol and phospholipid can equilibrate with the labelled cholesterol of rat erythrocyte ghosts within a period of 24 hours at 37°C without any net transfer of cholesterol in or out of the membrane (146). This implied that the cholesterol content of the erythrocyte ghost was dependent on the concentration of cholesterol present in its surrounding and that all of the erythrocyte cholesterol was accessible to the liposomal cholesterol. A similar conclusion regarding the exchangeability of membrane cholesterol was arrived at for a number of other subcellular organelles including liver plasma membranes, kidney microsomes, liver smooth endoplasmic reticulum and liver mitochondria when the distribution of labelled cholesterol contained in plasma lipoproteins was analysed after a 37°C incubation of lipoprotein with cell fraction (149).

The cholesterol exchange process is thought to be dependent on a number of factors (a) the nature of the fatty acids in the liposomes undoubtedly influences the exchange or the removal of cholesterol from the membrane. Poznansky and Lange (150) and Bloj and Zilversmit (151) among others have studied this aspect of the exchange process and although their results differ in absolute terms they both show that cholesterol containing liposomes prepared from unsaturated phospholipids allow a much higher rate of exchange

into the erythrocytes than liposomes prepared from unsaturated phospholipids. The nature of the fatty acyl chains has no effect on <sup>(a)</sup> the total amount of the exchangeable cholesterol in the erythrocyte; (b) the ratio of liposomal cholesterol to membrane cholesterol; (c) the existence of a non-exchangeable pool of liposomal cholesterol. Bruckdorfer et al. (146) and Bloj and Zilversmit (151) do not subscribe to the view of Poznansky and Lange (150) that in vesicles of dipalmitoylphosphatidylcholine and cholesterol only 70% of the cholesterol is available for exchange with the cholesterol found in the erythrocyte ghost. Poznansky and Lange (152) account for the inability of 30% of the labelled cholesterol to exchange by stating that it is located in the inner membrane of the liposome and is thus unavailable for exchange. This implies that the movement of lipid molecules from one side of a lipid bilayer to the other (flip-flop) (153) is slower than had been previously believed. This seems unlikely. Perhaps the observed differences in the rate of exchange can be rationalised in terms of differences in the sonicated liposomes used for the exchange. This explanation would be consistent with the data of Bloj and Zilversmit (151) who showed that the rate of radioactive cholesterol exchange from multilayered liposomes into the erythrocytes was 2.5 times slower than the exchange from unilamellar liposomes.

Another use of liposomes is to remove cholesterol from membranes in order to discover what function the cholesterol is performing. When erythrocyte ghosts were incubated with sonicated egg yolk phosphatidylcholine or sonicated egg yolk phosphatidylcholine containing 10 mole % cholesterol (the ratio of liposomal cholesterol to erythrocyte cholesterol is 0.1) after 24 hours the membranes

lost 32-46% of their sterol. When an excess of the dispersion was present, the ghosts lost 60% of cholesterol. A similar picture was also obtained by Shattil et al. (148) using liposomes to alter the cholesterol level in platelets. Platelets were incubated for 5 hours at 37°C with sonicated dipalmitoylphosphatidylcholine containing (a) equimolar amounts of cholesterol (b) a two-fold excess of cholesterol or (c) no cholesterol. The liposomes containing equimolar amounts of cholesterol and dipalmitoylphosphatidylcholine had no effect on platelet lipids or platelet function, whereas the platelets incubated with liposomes containing excess cholesterol acquired 39.2% excess cholesterol while the protein and phospholipid values remained unaffected. Dipalmitoylphosphatidylcholine liposomes removed 21% of the platelet cholesterol which resulted in an 18-fold reduction in their sensitivity to adrenaline (39.2% excess cholesterol caused a 35 fold increase in sensitivity to adrenaline). Thus using this technique it is possible to elucidate the role that cholesterol is playing in the platelet. A similar set of experiments suggested that cholesterol in erythrocytes was acting to strengthen the erythrocyte membrane (154).

The results obtained for the cholesterol depletion experiments have been explained by Lange and Alessandro (155). The exchange of cholesterol between erythrocytes and plasma and the net movement of cholesterol out of the membrane into the plasma are both characterised by the same rate constant, and are driven by the cholesterol to phospholipid ratios in the cells and plasma. The apparent limitation on the extent of cholesterol depletion of membranes is thus explicable as the consequence of an equilibrium between membrane

cholesterol and plasma cholesterol which is reached before all the cholesterol is removed from the membrane. It is proposed that the exchangeable cholesterol is available for removal from the membrane and thus there is only a single pool of erythrocyte cholesterol. Thus the limitation on cholesterol depletion of the erythrocyte does not imply the existence of a class of strongly bound cholesterol molecules. Rather it is due to the dissipation of the gradient which leads to net movement of cholesterol out of the cells into the plasma. Cholesterol exchange still occurs at equilibrium, between cells and plasma leading to the equilibration of radioactive cholesterol, between the two cellular fractions.

## 7.2 Preparation of liposomes for use in the cholesterol exchange and cholesterol depletion experiments

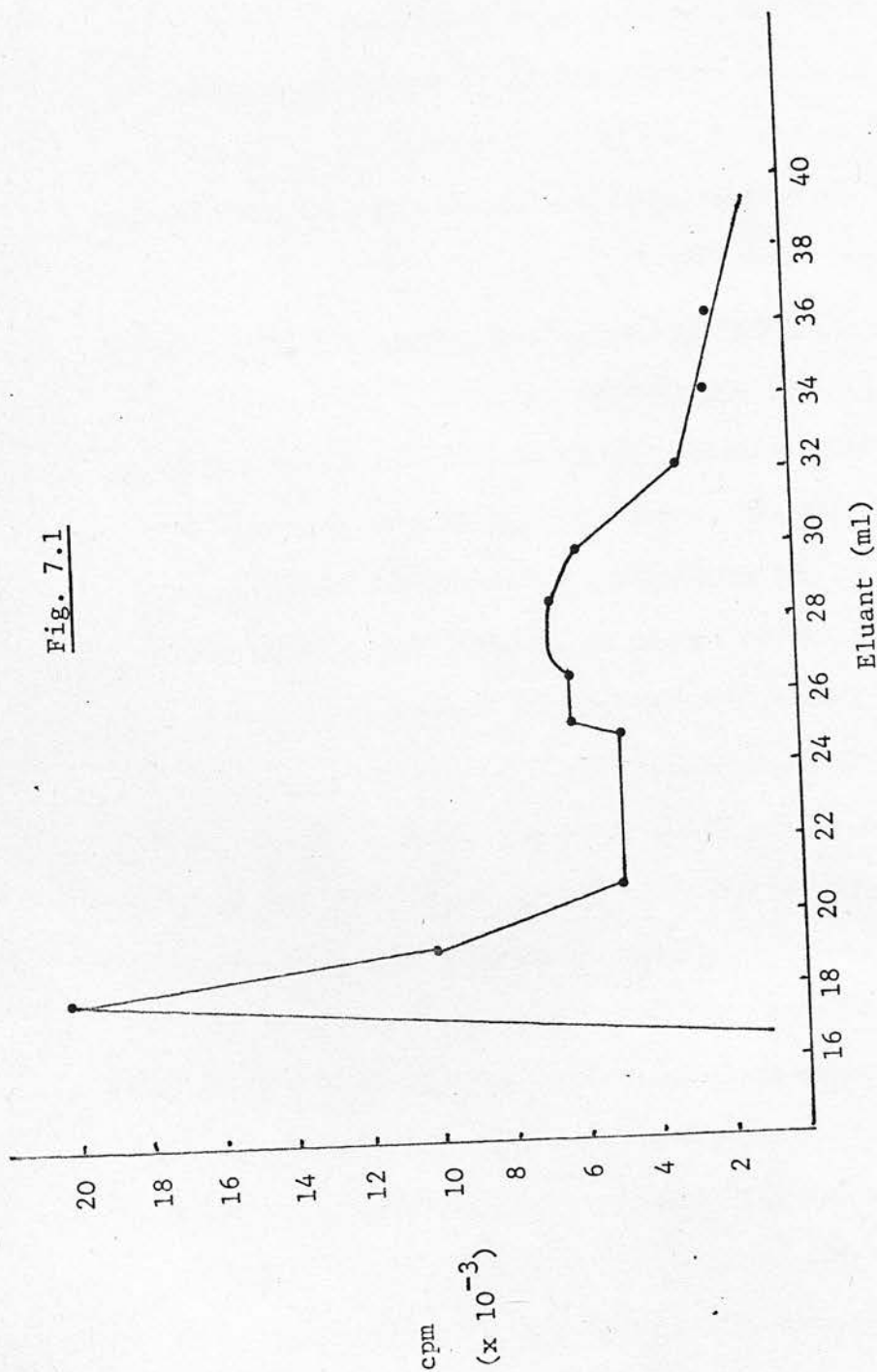
In the previous section differences in results of different groups working with the same biological systems was attributed to differences in the preparation of the liposomes used to deliver the label or to remove cholesterol from the membrane under study. The various methods for liposome preparation described in literature are

(1) Bruckdorfer et al. (154) prepared liposomes from dried films of egg yolk phosphatidylcholine (20 mg) and sterol (16 mg) by sonicating the lipid in 10 ml of 30 mM NaCl with a Branson Sonic Power Instrument Model S125 set at position 4. During the sonication procedure the glass vessel was cooled with an ice water mixture. The dispersions were then centrifuged at 48,000 g for 1 hour to remove the undispersed lipid which formed 30% of the mixture.

(2) Stevens and Green (156) found that a 5 x 90 sec burst of sonication interspersed by 20 second cooling periods was sufficient



Fig. 7.1



A typical sepharose 4B elution profile of egg yolk phosphatidylcholine liposomes prepared by sonicating the lipid dispersion (2mg/ml) for 1 x 90 secs.



to disperse a 1.6 mg/ml (1:1) mixture of sterol and phospholipid.

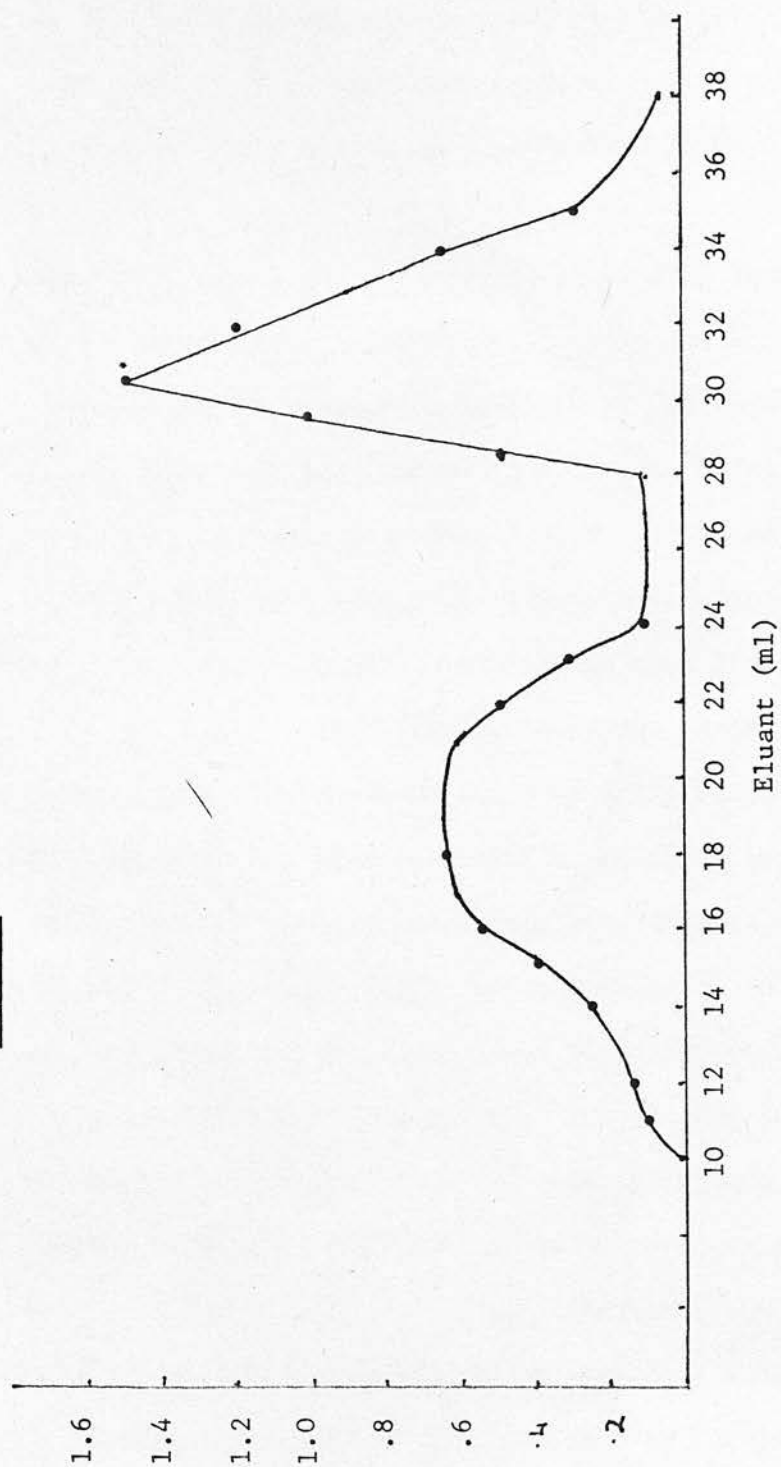
(3) The description of sonication conditions by Shattil et al. (148) is sketchy. They prepared liposomes by sonicating the lipid film in 10 ml of buffer for 1 hour with a 70W output sonicator.

(4) The method of Horwitz et al. (157) gives a more detailed description of the sonication conditions. A vessel of 5.5 cm height and diameter 2.4 cm was used to contain an undisclosed amount of lipid which was then converted into liposomes by sonicating for 40 minutes with a sonicator that operated at 20 kilo-cycles per second with a power output of 75W. In addition they found that the nature of the phospholipid fatty acyl chains influenced the uptake of cholesterol.

(5) Green and Green (158) used the sonication technique of Stevens and Green (156) but altered the ratio of lipid to water in order to see if there was a relationship between this ratio and the amount of lipid dispersed. With ratios above 6 mg/ml the maximum incorporation of cholesterol was 1:1 even when the initial ratio of cholesterol to phospholipid was 3:1. At a slightly lower concentration a 2:1 ratio could be obtained and if the starting mixture contained less than 1 mg lipid per ml all the sterol was dispersed.

As can be seen each sonication procedure has slight differences from other published methods and many factors can influence the nature of the liposomes produced by these various techniques. Hauser (159) discussed the differences and stated that the following factors were important. The ratio of the sample volume to the soniprobe tip was important. A one minute exposure of 2 ml of dispersion led to detectable decomposition,

Fig. 7.2



The sepharose 4B elution profile of liposomes prepared by sonicating a phospholipid dispersion by the method described in Chapter 2.

whereas 10 ml of dispersion gave degradation products only after 40 minutes of sonication. Besides the instrumental settings (power output, tuning of the probe and the sonication time) the geometry of the tip of the soniprobe relative to that of the sample tube, the volume, concentration, liquid depth, and temperature of the dispersion, the depth to which the top of the soniprobe was immersed, the characteristics of the lipid species involved and the nature of the dissolved gas and the atmosphere surrounding the sample were all found to influence the extent of lipid decomposition.

Obviously these sources of variation make comparison between different methods of liposome preparation extremely difficult. A possible method for further characterisation of the liposomes formed by the various sonication procedures was to compare the relative elution positions of liposomes upon molecular sieve chromatography. This was originally suggested by Huang (64) and has been used by Bloj and Zilversmit (151) among others to prepare homogeneous preparations of single walled liposomes suitable for use in exchange experiments.

The initial method of liposome preparation adopted was the procedure described by Stevens and Green (156). Egg yolk phosphatidylcholine (50 mg) was dispersed in 0.1 M phosphate (5 ml) buffer pH 7.4 by vortexing the lipid for 4 minutes. This was followed by sonication of the dispersion contained in 4.5 cm height by x 2.5 cm diameter vessel with a Branson sonicator, equipped with a 2 mm by 6 cm titanium probe, maintained at power level 7. The sonication consisted of 5 x 90 seconds periods interspersed with 20 second cooling periods. The resulting milky suspension was centrifuged at 74,000 g for 20 minutes and

the opalescent supernatant separated from the undispersed material which formed a white pellet. The supernatant was then applied to a Sepharose 4B column (1.5 cm x 33 cm) when all the material was seen to appear in the void volume. When the procedure was repeated using the same apparatus, but with double the period of sonication and 20 mg of egg yolk phosphatidylcholine in 10 ml of buffer as opposed to 50 mg in 5 ml, a similar result was obtained. If a trace of radioactive cholesterol was added to the lipid film prior to sonication under the second set of conditions, 69% of the label was recovered in the supernatant and the remaining 31% remained in the pellet. A typical elution profile of radioactive liposomes produced by this sonication procedure is given in Figure 7.1 which shows that virtually all of the label is located in a fraction of the lipid that appears in the void volume. A similar picture was obtained when the vortexed liposomes were placed in a sonicating bath similar to that described by Huang (64) and sonicated at maximum power setting for 3 hours. Thus neither of the sonication procedures seems to produce single walled liposomes from the multilayered liposomes formed by vortexing the dried lipid film in buffer.

Small single walled liposomes were eventually obtained by following the procedure described in Chapter 2. After 40 minutes of sonication the liposomal solution became optically clear and on centrifugation only titanium fragments from the sonicator tip centrifuged down. Figure 7.2 shows a typical elution profile of liposomes prepared in this manner, from the Sepharose 4B column. Although only 56% of the liposomal population appeared in the retained fraction which is 10% less than that in the published figures of Huang (64) it was used

14 hour incubation 37°C			14 hour incubation 4°C		14 hour incubation 37°C + E.Y.PC	
gradient density (gm cm <sup>-1</sup> )	chol (μg)	protein (mg)	chol. (μg)	protein (mg)	chol. (μg)	protein (mg)
1.176	0	0.133	16	0.386	6	0.197
1.161	3.6	0.059	8	0.089	0	0.104
1.151	3.6	0.104	8	0.059	0	0.104
1.137	0	0.059	0	0	0	0.114
1.127	5	0	0	0.208	0	0.044
1.113	3	0.089	5.2	0.029	0	0.074
1.104	0	0	0	0.044	0	0.074
1.084	3	0	5.3	0.176	0	0.059
1.081	0	0.118	0	0.118	0	0.089
1.068	3.6	0.089	8	0.236	0	0.148
1.059	7.2	0.236	14	0.564	0	0.252
1.047	0	0.743	0	0.564	6	0.743
1.038	0	0.921	27	1.009	20	1.069
Pellet	140	3.8	90	2.820	120	3.8

Continuous sucrose density gradient profile of microsomes incubated at either 4°C or 37°C in the presence or absence of phospholipid. Recovery >97%.

Table 7.1



without further subfractionation. Thus the sonicated liposomes prepared from egg yolk phosphatidylcholine are a mixture of large and small vesicles. The proportions of each type present in each preparation will differ slightly depending on the efficiency of the sonication. The incorporation of cholesterol analogues in a 1:1 or 1:10 ratio with the egg yolk phosphatidylcholine has no effect on the basic profile shown in Figure 7.2 when 3 mg lipid are dispersed in 1 ml of buffer.

### 7.3 Alteration of cholesterol levels in liver microsomes by treatment with egg yolk phosphatidylcholine liposomes

In an attempt to estimate what fraction of the exchangeable microsomal cholesterol pool described by Graham and Green (149) could be removed by egg yolk phosphatidylcholine liposomes, a series of preliminary experiments were performed using the techniques described for the removal of cholesterol from the erythrocyte (154). Basically this involved incubation of the microsomes with liposomes for various periods of time at 37°C and centrifuging the incubation mixture at 100,000 g for 1 hour to separate liposomes from microsomes. When the microsomal fraction was prepared in this way the liposomes did not appear to be having any noticeable effect on the microsomal cholesterol levels. A possible explanation of this result was that the unsonicated liposomes used in the experiments were co-sedimenting with the microsomes and were contributing to the measurement of cholesterol in the microsomal pellets. This problem was partly resolved by placing the incubation mixture of microsomes and liposomes on a sucrose density gradient. Both continuous gradients varying from a median density of  $1.038 \text{ g cm}^{-1}$  to a median density of  $1.176 \text{ g cm}^{-1}$  (10-40% w/v) and discontinuous gradients



E.Y.PC (mg)	protein (mg)	chol. ( $\mu$ g)	P.L (mg)	chol/P.L.	chol/protein
0	2.4	118	1.20	0.098	0.049
3 (a)	1.92	118	1.32	0.089	0.046
6	2.3	91	1.45	0.062	0.039
9	2.3	76	1.16	0.062	0.033
0 (b)	6	120	1.25	0.098	0.020

An analysis of the 100,000g pellets formed by the 4°C overnight incubation of microsomes with E.Y.PC followed by sucrose density gradient centrifugation. (b) shows an analysis of the microsomal preparation. (P.L. = phospholipid)

Table 7.2

Incubation conditions	chol ( $\mu$ g)	protein (mg)	P.L (mg)	chol/P.L.
4°C	169 $\pm$ 5	4.29 $\pm$ 0.5	1.25 $\pm$ 0.2	0.134
4°C + E.Y.PC (a) (4mg)	100 $\pm$ 5	3.47 $\pm$ 0.5	0.95 $\pm$ 0.2	0.105
37°C + E.Y.PC (4mg)	70 $\pm$ 5	3.38 $\pm$ 0.5	1.10 $\pm$ 0.2	0.063
(b)	180 $\pm$ 5	8.0 $\pm$ 0.5	1.32 $\pm$ 0.2	0.136

Analysis of the 100,000g pellets formed after sucrose density gradient centrifugation. (b) describes the microsomal preparation used in the incubation. Results are the means of 6 determinations  $\pm$  standard deviation.

Table 7.3

(20,30,60% w/v) (160) were used for the separation of liposomes from the membrane fractions. In this system the lipid floats on the surface of the sucrose and the various microsomal components were separated on the basis of their relative sizes. Table 7.1 shows that overnight incubation, at 37°C or 4°C, of unsonicated liposomes with a reconstituted freeze dried powder of microsomes (containing 6.6 mg protein and 170 µg cholesterol) gave a reduction in the absolute amount of cholesterol present in the 100,000 g pellet. However there was no change in the cholesterol:protein ratio. Table 7.1 also shows that the microsomal protein spreads throughout the sucrose gradient with slight differences in the exact distribution which can be attributed to temperature effects.

If the amount of cholesterol in the 100,000 g pellets is related to the amount of phospholipid present which is unaffected by the liposomal phospholipid or 37°C incubation then the results are slightly different. Table 7.2a shows that even at 4°C when the results are expressed in relation to the phospholipid, liposomal phosphatidylcholine removed 36% of the cholesterol upon overnight incubation (Table 7.2b is an analysis of the microsomal preparation that was incubated with the liposomes). After a 14 hour incubation at 37°C the microsomal suspension changes to a light granular suspension indicating that the membranes have been altered by the incubation procedure.

When the experiments were repeated using sonicated egg yolk phosphatidylcholine liposomes in a 40 fold excess over the microsomal cholesterol the removal of cholesterol improved. Table 7.3 shows 100,000 g sucrose density gradients pellets obtained from four experiments contain similar amounts of protein

protein (mg)	chol. ( $\mu$ g)	P.L. (mg)	chol/P.L.	chol/protein
(a) 6	100	1.92	0.052	0.0167
(b) 2.5	80	1.85	0.043	0.032
(c) 2.46	180	2.16	0.083	0.073

Analysis of the 100,000g pellets formed by a 37°C overnight incubation of the microsomes (b) alone, (c) with liposomes containing a four fold excess of cholesterol. (a) represents an analysis of the microsomal preparation.

Table 7.4

fraction 1=top	cytochrome P450 nmole		protein ( mg )	
	14 hour 4°C	14 hour 4°C +E.Y.PC	14 hour 4°C	14 hour 4°C +E.Y.PC
1	0	0	0.70	0.87
2	0	0	0.80	0.70
3	0	0	0.92	1.12
4	0.24	0.4	0.87	1.05
5	0.4	0.5	0.87	1.02
6	0	0	0.52	0.29
7	0	0	0.87	0.41
8	0	0	0.87	0.85
9	1.4	1.8	3.37	3.37

Distribution of microsomal cytochrome P450 and protein in a 20,30,60% discontinuous sucrose gradient. The microsomal suspension contained 9.64 mg protein and 4 nmole cytochrome P450.

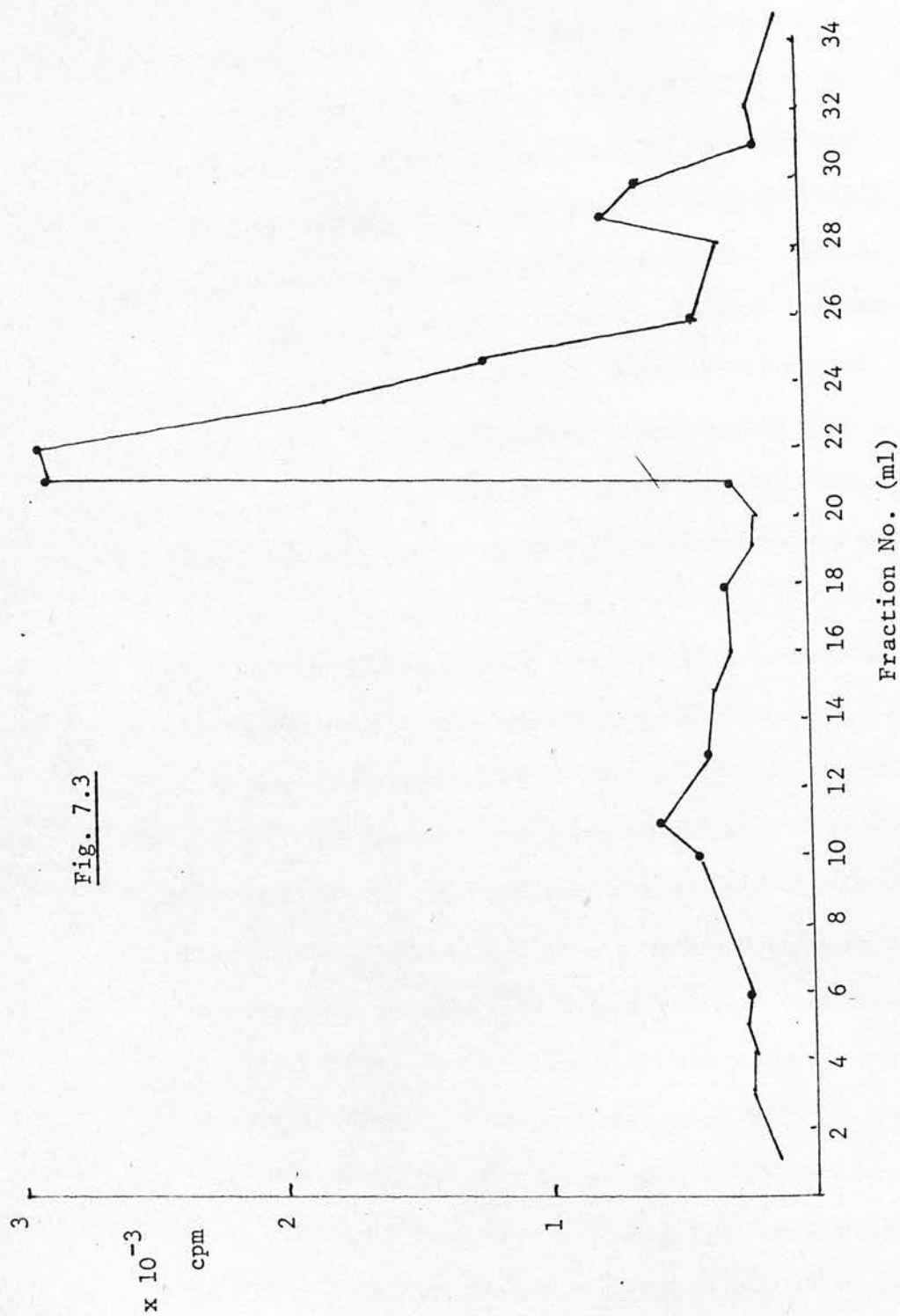
Table 7.5

and phospholipid to those obtained before. The amount of cholesterol removed increases with increasing incubation temperature and the maximum reduction that was achieved was 52% of that in the intact microsome when measured relative to the microsomal phospholipid.

Since these results showed that cholesterol can be removed from microsomes by incubation with egg yolk phosphatidylcholine, it was decided to try to increase the amount of cholesterol in the microsomes by incubating them with cholesterol containing liposomes. Unsonicated liposomes were prepared from cholesterol (4.2 mg) and egg yolk phosphatidylcholine (10 mg) and an aliquot containing 400  $\mu$ g of cholesterol was incubated with a microsomal suspension containing 100  $\mu$ g cholesterol. Table 7.4 shows that the microsomes can increase their cholesterol complement by 60% under these incubation conditions.

The treatment of microsomes with liposomes of varying compositions seems to be a technique that should allow the microsomal pool of cholesterol to be manipulated using mild conditions. Unfortunately there are problems associated with the methodology which as yet have not been completely solved. The major problem concerns the fact that the microsomes sub-fractionate when they are placed on a sucrose gradient and thus a better method for separating the liposomes from the microsomes will have to be developed. A sucrose medium of some sort may still be required as even single walled liposomes have a tendency to aggregate (159) and thus to sediment with the microsomes. In addition the overnight incubation may have a deleterious effect on the enzymic activity of the microsomes.

Fig. 7.3



Sepharose 4B elution profiles of  $^3\text{H}:\text{C}_{24}$ : egg yolk phosphatidylcholine (1:1)  
liposomes.



Thus the methodology involved in trying to alter the cholesterol levels in the microsome is more complicated than that published for the erythrocyte and is still not entirely satisfactory.

#### 7.4 The effect of egg yolk phosphatidylcholine on the activity of the microsomal cholesterol 7 $\alpha$ -hydroxylase

In the previous section it was shown that up to 52% of the microsomal cholesterol expressed as a cholesterol:phospholipid ratio can be removed by treatment with egg yolk phosphatidylcholine. In addition to check if this technique could be adopted for the study of cholesterol 7 $\alpha$ -hydroxylase the properties of a microsomal preparation which had been treated by egg yolk phosphatidylcholine were investigated. Fresh microsomes containing 9 mg of protein and 4 nmoles cytochrome P450 were incubated at 4°C for fourteen hours in the presence or absence of egg yolk phosphatidylcholine. Table 7.5 shows the 20, 30, 60% (w/v) discontinuous sucrose density gradient profile of cytochrome P450. Only 50% of the cytochrome P450 added to the gradients was recovered. As can be seen in both cases 66% of the recovered cytochrome P450 was found in the fraction that floats on top of the 60% sucrose cushion. In addition cytochrome b<sub>5</sub> and NADPH cytochrome c reductase were located in the pellet fraction alone and were recovered in 80-90 per cent yield. No cytochrome P420 was detected. The remaining cytochrome P450 found at the boundary between the 20 and 30% sucrose layers did not support 7 $\alpha$ -hydroxylation. Thus egg yolk phosphatidylcholine removes cholesterol from the microsome without destroying the basic microsomal organisation, under the described incubation conditions.



The cholesterol 7 $\alpha$ -hydroxylase activity of these pellets with reduced cholesterol levels was determined to see if enzyme activity was retained after overnight incubation at 4°C and a subsequent sucrose density gradient centrifugation. When the results are expressed as the percentage conversion to 7 $\alpha$ -hydroxycholesterol per mg pellet protein, the results showed that the enzyme activities of both the treated and untreated microsomes were not significantly different. If the microsomes were incubated at 37°C as stated previously this resulted in a 50% depletion of the microsomal cholesterol. Unfortunately the procedure also destroyed the cytochrome P450 and eliminated the cholesterol 7 $\alpha$ -hydroxylase activity.

The activity of the cholesterol 7 $\alpha$ -hydroxylase as measured by the tracer assay is not altered by removal of 36% of the microsomal cholesterol. Perhaps this result is not altogether unexpected as a fully depleted preparation of microsomes (butanol powder) still retains the cholesterol 7 $\alpha$ -hydroxylase activity.

#### 7.5 Comparison of the abilities of the side chain analogues to exchange with microsomal cholesterol pool

As previously noted the erythrocyte has been the membrane of choice for study of the role of cholesterol in membranes. Using erythrocyte ghosts and liposomally bound steroids containing 2 to 3 times the weight of cholesterol in the erythrocyte. Bruckdorfer et al. (154) were able to show that 30% of 7-dehydrocholesterol, lathosterol,  $\beta$ -norcholesterol and cholest-4-en-3-one could exchange with erythrocyte cholesterol. The failure of other sterols to exchange was attributed to their poor incorporation into a liposome. There is no record of a similar study in the liver microsomes where the cholesterol present is

Fraction		c.p.m.
incubation residue		10,950
sucrose gradient residue		2,464
100,000g centrifugation res.		10,645
100,000g supernatant		63,000
100,000g pellet		585,690
sucrose gradient :		
Top	1	105,888
	2	23,845
	3	10,511
	4	9,544
	5	16,951
	6	13,928
	7	10,247
	8	3,702

Typical distribution of radioactivity in the various fractions involved in the experimental procedure.  $1 \times 10^6$  c.p.m. were added and 87% was recovered.

Table 7.6

participating in many cellular processes including the production of steroid hormones and bile acids. As lipoprotein bound cholesterol has been shown to exchange with smooth endoplasmic reticulum (149) it was decided to find out if liposomal cholesterol was also capable of exchanging with microsomal cholesterol.

A microsomal suspension (1 ml) containing approximately 5 mg of protein and 100  $\mu$ g of cholesterol was incubated for various intervals at 37°C with sonicated liposomes containing a similar amount of radioactive cholesterol prepared from lipid films containing tritiated cholesterol and egg yolk phosphatidylcholine in a 1:1 molar ratio. The sonication procedure used, incorporated 80-100% of the radioactive cholesterol into a liposomal structure. Figure 7.3 shows a typical elution profile of the radioactive liposomes prepared in this manner. The profile is that of liposomes containing equimolar quantities of the C<sub>24</sub> analogue and phosphatidylcholine but a similar profile was obtained with each of the cholesterol analogues. The exact elution volume was varied between 24 and 31 ml depending on the precise tuning of the sonication. In some of the initial experiments the liposomes contained [<sup>14</sup>C]-glycerol trioleate which Zilversmit and Hughes (161) stated to be a non-exchangeable marker in a mixture of microsomes and liposomes. In my hands this marker compound exchanged into the microsomes rendering this method of measurement of the exchange useless.

After the incubation period, the incubation mixtures were placed on top of 20 (3 ml), 30 (4 ml) and 60 (4 ml) % (w/v) discontinuous sucrose density gradients prepared in 10 mM Tris, 1 mM EDTA pH 7.4 which were then centrifuged at 100,000 g for

Time (hr)	% exchange	protein	cholesterol	P.L.
		(mg)	( $\mu$ g)	(mg)
0	0	2.42	100	1.40
1	34 $\pm$ 2	2.48	100	1.52
2	40 $\pm$ 2	2.6	100	1.34
3	45 $\pm$ 3	2.6	100	1.42
7	70 $\pm$ 5	3.23	120	1.54
14	60 $\pm$ 5	2.89	110	1.46

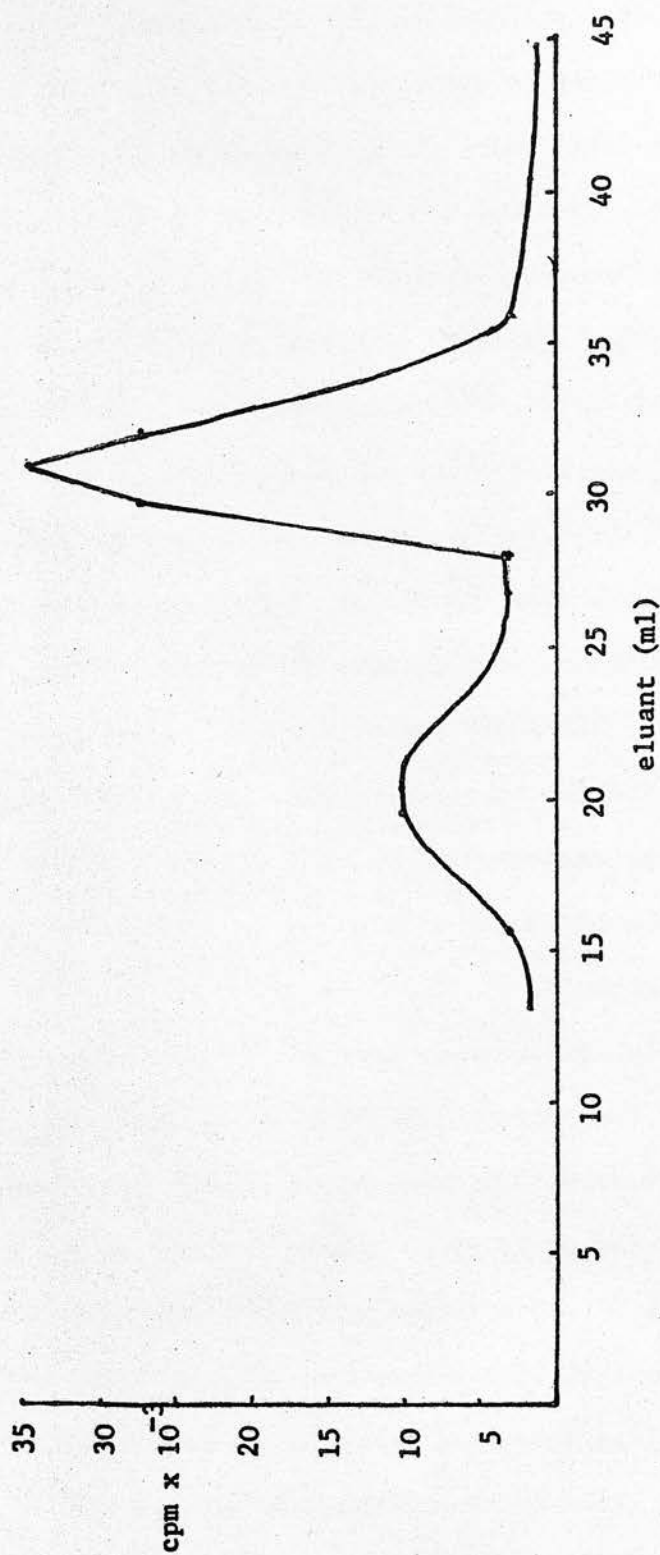
Time course of cholesterol exchange into microsomes when cholesterol is presented in a liposome containing equimolar amounts of cholesterol and phospholipid. The microsomal and liposomal membranes contain equivalent amounts of cholesterol.

Table 7.7

1 hour. The gradient was divided into 1 ml fractions and the material that floated on top of the 60% sucrose cushion was isolated, diluted with buffer and recentrifuged at 100,000 g for 1 hour in order to determine what proportion of the added radioactivity was tightly bound to the microsomal fraction. The pellet thus obtained was resuspended in buffer and the radioactivity, protein, cholesterol and phospholipid present were determined. The only factor that was found to alter was the radioactivity, indicating that cholesterol exchange was occurring and that there was no movement of sterol. The radioactivity value was corrected for losses that occurred during the manipulation and the ability of a liposomal cholesterol analogue to exchange with microsomal cholesterol was expressed as a percentage of the added radioactivity. Table 7.6 shows a typical distribution of radioactivity through the fractions involved in the experimental procedures when liposomes containing  $C_{24}$  and phosphatidylcholine in a (1:10) molar ratio were incubated at 37°C for 14 hours.

The initial experiments established that after 14 hours of incubation of cholesterol containing liposomes (cholesterol: phospholipid 1:1) with microsomes, the maximal exchange of radioactivity had occurred. Table 7.7 gives an indication of the time scale of the exchange process. The value of the amount of endoplasmic reticulum cholesterol available for exchange is considerably less than that published by Graham and Green (149) who incubated liver smooth endoplasmic reticulum with a plasma lipoprotein fraction containing the radioactive cholesterol. , The difference may be due to the ability of the lipoprotein to deliver cholesterol to the microsomes at a faster rate than liposomes. That the difference was due to differences between

Fig. 7.4



Sephacrose 4B elution profile of  $^3\text{H}$  C<sub>21</sub>:egg yolk phosphatidylcholine  
(1:10) liposomes.



Sterol	sterol:E.Y.PC (1:1)		sterol:E.Y.PC (1:10)	
	% sterol in liposome	% exchange into microsomes	% sterol in liposomes	% exchange into microsomes
C <sub>21</sub>	94	37	85	41±5
C <sub>24</sub>	97	52±5	87	59±8
C <sub>27</sub>	95	65±5	94.6	65±5
C <sub>28</sub>	97.5	36.5±5	88	35±5
	(a)	(b)	(a)	(b)

The effect of altering the cholesterol side chain and the cholesterol:phospholipid ratio on the exchangeability of cholesterol analogues with microsomal cholesterol. Column(a) represents the efficiency of incorporation of each analogue into a liposome , and the exchange is measured after a 14 hour incubation at 37°C.

Table 7.8

Sterol	% incorporation of sterol into liposomes	% exchange into microsomes.
C <sub>21</sub>	32	38±5
C <sub>24</sub>	67	62±5
C <sub>27</sub>	54	58±5
C <sub>28</sub>	68	39±5

The exchange of cholesterol analogues contained in dimyristoyl phosphatidylcholine, in a 1:10 molar ratio, with an equivalent amount of microsomal cholesterol.

Table 7.9

liposome and lipoprotein was confirmed when the relative transport abilities of sonicated and unsonicated liposomes <sup>were compared. Sonicated liposome</sup> transferred 60% of the radioactivity, whereas unsonicated liposomes only transferred 16%.

As the preliminary experiments had shown that the developed procedure could deliver radioactivity into the microsomes, the ability of three side chain analogues to exchange with microsomal cholesterol was investigated. Each of the cholesterol analogues tested can be incorporated in liposomes in a 1:1 ratio (162). Thus unlike Bruckdorfer (154) the liposomes did not require a proportion of cholesterol to ensure that the sterol and phospholipid are present in equimolar amounts. In each experiment the liposome and microsome contained equimolar amounts of cholesterol or cholesterol analogues, which ensured that no net movement of cholesterol would occur. In the exchange experiments already quoted, the ratio of liposome cholesterol to membrane cholesterol varied from 1:32 (151) to 1:50 (152). This prevented net movement of cholesterol but perhaps is a slightly artificial situation.

The microsomal exchange experiments were repeated with liposomes containing the molar proportions of cholesterol analogue and phospholipid found in the microsomes (0.1 mole cholesterol per mole phospholipid). This ensured that any exchange occurring was measured under equilibrium conditions. Table 7.8 shows that more than 80% of the radioactivity added to the lipid film is present in the liposomal fraction. Furthermore it also shows that in both liposomal preparations approximately 60% of the  $[^3\text{H}]\text{C}_{24}$  and cholesterol  $\text{C}_{27}$  was freely

exchangeable with the microsomal cholesterol, whereas only 40% of the  $C_{21}$  and  $C_{28}$  exchanged. As each sterol was equally incorporated into a liposome and the Sepharose 4B profiles (Figure 7.4) indicated that the sonication was equally effective in the cases of all the analogues (including  $C_{21}$ ) then this implies that the exchange process was regulated at the microsomal level.

When the liposomes were prepared from dimyristoyl phosphatidylcholine, the amount of radio-labelled sterol incorporated into the sonicated liposomes was reduced compared with the unsaturated phospholipid (Table 7(9)). However when the sterol exchanged into the microsomes was expressed as a percentage of the total sterol present a similar pattern to that previously observed was noted. This is contrary to the picture presented by Bloj and Zilversmit (151) that the rate of cholesterol transfer was proportional to the degree of unsaturation of the lipids comprising the liposome for cholesterol exchange into a red cell.

The results in this section indicate that when cholesterol is presented to rat liver microsomes in liposomes of different composition, the labelled cholesterol molecule can exchange with the microsomal cholesterol. Furthermore the side chain of cholesterol appears to be playing a role in determining the rate of cholesterol exchange process as  $C_{24}$  and cholesterol exchange with microsomal cholesterol more readily than  $C_{28}$  or  $C_{21}$ .

## 7.6 Conclusion

The work involved in this chapter has been concerned with trying to alter the microsomal cholesterol pool by incubating the microsomes with liposomes prepared from pure egg yolk

phosphatidylcholine, or with liposomes containing the available cholesterol side chain analogues that are radioactively labelled. The methodology involved the use of discontinuous and continuous sucrose density gradients to separate the liposomes from the microsomal fraction. In addition to separating these two components the sucrose also subfractionated the microsomes which is a recognised phenomenon (163). The liver microsomal fraction results when the supernatant fluid from the mitochondrial pellet is recentrifuged at 100,000 g. For maximal recovery of liver microsomes, extensive homogenisation at high speed is required to disrupt as many of the hepatocytes as possible but such homogenisation also results in increased damage to and fragmentation of cellular organelles. Thus the precise conditions used in the homogenisation such as the speed of the pestle and the number of passes performed during homogenisation determine the extent of the contamination of the microsomal fraction by plasma membrane, golgi cisternae, connective tissue cells, outer mitochondrial membranes or lysosomes. As these details are not always comprehensively described it is difficult to compare different microsomal preparations.

Analysis of the continuous sucrose density gradients showed that the main microsomal fraction has a median density greater than or equal to  $1.176 \text{ gm cm}^{-3}$  and contained cholesterol  $7\alpha$ -hydroxylase activity, cytochrome  $b_5$  and P450, phospholipid and at least 80 per cent of the microsomal cholesterol. The proportion of added protein found in the fraction was dependent on the experimental conditions.

The cholesterol levels in the main microsomal fraction were altered by incubation with liposomes of varying compositions. The results showed that the microsomes could be enriched with cholesterol by treatment with cholesterol containing liposomes. This was not achieved in Chapter 6 when butanol powders were incubated with acetone solution of cholesterol, implying that the butanol treatment abolished the ability of the microsomes to sequester increased levels of cholesterol. In addition the microsomal cholesterol was replaced by radioactive cholesterol side chain analogues presented in liposomes.

However, after a fourteen hour incubation at 4°C of microsomes with liposomes, when approximately 30% of the microsomal cholesterol was removed, addition of tracer cholesterol to the fraction showed that the activity of the cholesterol 7 $\alpha$ -hydroxylase enzyme was unaffected. This was not unexpected as butanol treatment of the microsomes depleted the microsomal cholesterol but the cholesterol 7 $\alpha$ -hydroxylase activity was retained as measured by the tracer assay. Another possible explanation was that the cholesterol removed was not providing substrate for the enzyme.

This chapter has also shown that a cholesterol analogue contained in a liposome can exchange with 60% of the microsomal cholesterol. This process required a 37°C 14 hour incubation, which destroyed the enzyme activity and thus it was not possible to see if the exchanged cholesterol analogues were metabolised in a different way from that described in Chapter 6.

Although those experiments have not been able to show whether alteration of the microsomal cholesterol pool will alter the activity of the cholesterol 7 $\alpha$ -hydroxylase the fact that the



liposomal cholesterol pool is capable of exchanging with microsomal cholesterol implied that at least 60 per cent of the microsomal cholesterol could have been obtained from plasma lipoproteins. This figure for the amount of exchangeable cholesterol is similar to that noted by Balasubramaniam et al. (164) for the percentage of microsomal cholesterol that is immediately accessible to the enzyme.

Balasubramaniam also indicated that the two pools were mutually exclusive and that the preferred substrate for the cholesterol 7 $\alpha$ -hydroxylase was cholesterol synthesised in the microsome (164). Whether in fact the lipoproteins supply a source of substrate for the cholesterol 7 $\alpha$ -hydroxylase enzyme must remain a matter for conjecture due to the difficulty of experimental design.

#### Summary

- (1) The preparation of single bilayer liposomes suitable for delivery of sterols into microsomes is described.
- (2) The ability of liposomes of various compositions to alter the microsomal cholesterol pool is described.
- (3) The use of sucrose density gradients to separate the microsomes from the liposomes and an analysis of the effect of sucrose density gradient centrifugation on the microsomal components is outlined.
- (4) The effect of alteration of the cholesterol side chain on the ability of the liposomally bound sterol to exchange with microsomal cholesterol is described.



CHAPTER 8INVESTIGATION OF THE POSSIBLE INVOLVEMENT OF ASUPERNATANT PROTEIN IN RAT LIVER MICROSOMALCHOLESTEROL 7 $\alpha$ -HYDROXYLASE

## 8.1 Introduction

The liver microsomal fraction contains cholesterol, which is a structural component of membranes as well as an enzyme substrate. The possible sources of substrate cholesterol for cholesterol 7 $\alpha$ -hydroxylase have been much discussed in the literature in recent years. Balasubramaniam et al. (164) have shown that when [ $^{14}\text{C}$ ]-cholesterol was incubated with liver microsomes under conditions in which 7 $\alpha$ -hydroxylation takes place the specific activity of the 7 $\alpha$ -hydroxycholesterol formed during the incubation was lower than that of the added cholesterol. This showed that some of the endogenous cholesterol in the microsomes which was equilibrating with the exogenous radioactive cholesterol was accessible to the enzyme. They have proposed the term "substrate pool" for that fraction of the microsomal cholesterol that acts as substrate for cholesterol 7 $\alpha$ -hydroxylase and they estimated that approximately 70% of the microsomal cholesterol was accessible to the enzyme. This same figure was reached by Van Cantfort et al. (128) using different techniques and the same assumption that the exogenous cholesterol equilibrated completely with the microsomal cholesterol.

In the same paper Balasubramaniam et al. (164) isolated liver microsomes from rats shortly after they had been injected with [ $^{14}\text{C}$ ] cholesterol and noted that the specific activity of the 7 $\alpha$ -hydroxycholesterol formed by the microsomes was lower than that of the microsomal cholesterol. They concluded that the pool of cholesterol from which 7 $\alpha$ -hydroxycholesterol was synthesised does not equilibrate rapidly with plasma cholesterol and they therefore suggested that this pool was derived preferentially from cholesterol synthesised in the rough endoplasmic reticulum. This finding was

confirmed by Bjorkhem and Danielsson (11) who showed that the specific activity of  $7\alpha$ -hydroxycholesterol formed from  $[5-^3\text{H}]$  mevalonic acid during incubations of rat liver microsomes was higher than that of the total microsomal cholesterol.

Cholesterol feeding increases cholesterol absorption by the liver and decreases hepatic cholesterol synthesis as judged by the activity of HMG CoA reductase (165). In addition there is a slight increase in the excretion of endogenous neutral steroids but it is insufficient to prevent an accumulation of cholesterol in the liver. Under these conditions the percentage of cholesterol formed in the liver will obviously decrease. However when the cholesterol  $7\alpha$ -hydroxylase activity of the cholesterol fed animals was measured, some workers (9,132) observed an increase whereas others failed to notice any significant change from control values (11). The failure to observe any increase in formation of  $7\alpha$ -hydroxycholesterol could be rationalised by postulating that the enzyme was supplied by a pool of cholesterol that is largely unaffected by alteration of the general hepatic cholesterol pool. Alternatively the previously mentioned deficiencies of the tracer assay may prevent an increase in the activity of the enzyme from being detected.

The increase in the activity of cholesterol  $7\alpha$ -hydroxylase could be explained by an alteration of the source of cholesterol for the enzyme under these conditions. As cholesterol feeding inhibits hepatic cholesterol synthesis while at the same time increasing  $7\alpha$ -hydroxylation of cholesterol newly synthesised cholesterol cannot be the only source of substrate. Perhaps cholesterol feeding facilitates transfer of cholesterol that does not normally provide substrate for the enzyme from a pool supplied by dietary sources to a pool which normally supplies

cholesterol for hydroxylation.

The situation was further complicated by the findings of Gustafsson et al. (166) using germ free rats. Germ free rats have a higher level of liver cholesterol than "normal" animals and cholesterol synthesis is decreased. In normal rats bile acids are metabolised by intestinal microflora to a spectrum of secondary and non-steroidal derivatives, however in germ free rats only cholic acid and chenodeoxycholic acid are formed. When germ free rats were fed cholesterol, chenodeoxycholic acid production was stimulated to a greater extent than cholic acid and under these conditions the endogenous formation of cholesterol was decreased. <sup>Gustafsson</sup> explained the results by suggesting that the substrate pools of cholesterol for chenodeoxycholic and cholic acid formation were different, the cholic acid pool consisting of the same newly synthesised cholesterol proposed by Balasubramaniam et al. (1964) and the chenodeoxycholic acid substrate pool being freely available to the endogenous cholesterol.

It should be noted that the experiments described above showed only that cholesterol newly synthesised in the hepatic microsomes was the preferred substrate for cholesterol 7 $\alpha$ -hydroxylase and not that it was the sole substrate. All the exchangeable cholesterol in the body is potentially accessible to the enzyme (167). This is obvious from the well known observation that the bile acids become labelled if ring labelled cholesterol is injected intravenously. The situation revealed by observations on the relative specific activities of microsomal cholesterol under different experimental conditions may perhaps be expressed most simply by saying that the pool of cholesterol that provides substrate for cholesterol 7 $\alpha$ -hydroxylase equilibrates more rapidly with cholesterol molecules that have just been synthesised in hepatic microsomes than with molecules reaching the microsomes from other sources including lipoproteins from cholesterol fed animals.

Another possible method for regulating the activity of the enzyme by altering substrate supply would be by a specific transport mechanism for cholesterol from the cholesterol pools to the enzyme. The presence of a saturable carrier would be consistent with the possibility that the rate of formation of 7 $\alpha$ -hydroxycholesterol is regulated by the size of the enzyme's substrate pool.

The existence of a factor that would affect the activity of cholesterol 7 $\alpha$ -hydroxylase was first described by Mayer (168). Unfortunately the peptide that he isolated from rat liver 100,000 g supernatant inhibited the enzyme. More recently Spence and Gaylor (13) in a paper which was mainly concerned with the effect of a protein purified from 100,000 g supernatant on the activity of HMGCoA reductase indicated that a 40-80% ammonium sulphate treatment of rat liver 100,000 g supernatant also contained a protein that improved the conversion of cholesterol to 7 $\alpha$ -hydroxycholesterol. This finding would be consistent with the observation that other microsomal enzymes are stimulated by, or are dependent on, the presence of a cytosolic protein. They include the conversion of

- (1) squalene to lanosterol (169)
  - (2) lanosterol to dehydrolanosterol (170)
  - (3) lanosterol to C<sub>27</sub> sterol (171)
  - (4) dihydrolanosterol to cholesterol (172)
  - (5) 5-cholest-7-en-3 $\beta$ -ol to cholesta-5, 7-dien-3 $\beta$ -ol (173)
  - (6)  $\Delta^7$ -cholestenol to 7-dehydrocholesterol and of 7-dehydrocholesterol to cholesterol (174) (175)
  - (7) vitamin D<sub>3</sub> to 25-hydroxyvitamin D<sub>3</sub> (176)
- and (8) 7 $\alpha$ -hydroxy-4-cholesten-3-one to 7 $\alpha$ ,12 $\alpha$ -dihydroxy-4-cholesten-3-one (177) among others.

microsomal protein (mg)	cytosolic protein (mg)	B.S.A.	% recovery of labeled	% conversion
15	0	0	64	4 $\pm$ 0.5
15	90	0	67	7.9 $\pm$ 0.5
15	0	90	62	4 $\pm$ 0.5
0	90	0	66	0

A comparison of the abilities of equivalent amounts of bovine serum albumin (B.S.A) and unfractionated 100,000g supernatant to enhance the activity of cholesterol  $\alpha$ -hydroxylase. Results are the means  $\pm$  the standard error of the mean.

Table 8.1

microsomal protein(mg)	cytosolic protein(mg)	trypsin (mg)	% conversion
15	60	1	7.9 $\pm$ 0.4
15	60	5	8.0 $\pm$ 0.5
15	60	0	14.0 $\pm$ 0.5
15	0	0	9.0 $\pm$ 0.5

The effect of a trypsin treatment on the ability of unfractionated 100,000g supernatant to increase the activity of microsomal cholesterol  $\alpha$ -hydroxylase. The trypsin solution contained 10mM CaCl<sub>2</sub> and was incubated with protein for 12 hours.

Table 8.2



Treatment	cytosolic protein(mg)	% conversion
none	none	$3.6 \pm 0.3$
none	20	$5.0 \pm 0.2$
none	40	$6.0 \pm 0.3$
none	60	$6.7 \pm 0.3$
none	90	$7.0 \pm 0.5$

The effect of the addition of differing amounts of ammonium sulphate fractionated 100,000g supernatant to a lyophilised preparation of microsomes containing 15mg protein.

Table 8.3

Treatment	cytosolic protein(mg)	% conversion
none	none	$4.6 \pm 0.2$
1 min(80°C)	30	$9.1 \pm 0.2$
2 min(80°C)	30	$6.4 \pm 0.5$
5 min(80°C)	30	$6.0 \pm 0.3$
none	30	$8.0 \pm 0.1$

The effect of different periods of heat treatment on the stimulatory activity of an 40-80% ammonium sulphate fraction from rat liver 100,000g supernatant. The microsomal preparation contained 6mg protein.

Table 8.4

The precise role that these soluble proteins play in mediating the activity of the microsomal enzyme is a matter of conjecture. In a recent review Scallen et al. (178) implied that each soluble protein may be a soluble constituent of the particle-bound enzyme. The soluble component could thus fulfil both a carrier or binding role towards a water insoluble substrate and a catalytic role in conjunction with the membrane bound enzyme component. However Saat and Bloch (179) thought that this was too precise a definition of the role of the supernatant proteins and they surmised that they were more likely to be transporting exogenous or endogenous substrate to a specific site or compartment within the membrane. Furthermore as enzymes were associated with the endoplasmic reticulum they considered that the active proteins, although isolated from the 100,000 g supernatants, were in fact peripheral microsomal proteins. Since the microsomes contained numerous inter-related enzymes the existence of several "supernatant" proteins seems likely.

The following sections describe the initial attempts to confirm the findings of Spena and Gaylor (13) regarding the involvement of supernatant protein in microsomal cholesterol 7 $\alpha$ -hydroxylase.

## 8.2 Involvement of a cytosolic protein in cholesterol 7 $\alpha$ -hydroxylase

In order to check if the 100,000 g supernatant of rat liver possessed the ability to stimulate the activity of microsomal cholesterol 7 $\alpha$ -hydroxylase, the 100,000 g supernatant was obtained from cholestyramine fed (4% w/w), female rats and lyophilised. After reconstitution in phosphate buffer (0.1M pH 7.4) the supernatant fraction was added to a standard 7 ml incubation containing  $[4-^{14}\text{C}]$  cholesterol (described in chapter 2) at a supernatant protein to microsomal protein ratio of 6:1 and the reaction was incubated at 37°C for 1 hour. Following a lipid extraction and thin layer chromatography the amount of radioactivity

Treatment	Nature of product formed	% conversion
$\beta$ mercapto-ethylamine (10mM)	$7\alpha$ hydroxy-cholesterol	4.6
40-80% $(\text{NH}_4)_2\text{SO}_4$ fraction (30mg)	$7\alpha$ , $7\beta$ , hydroxy-cholesterol, 7 - keto cholesterol	4.0
$\beta$ mercapto-ethylamine, + 40-80% $(\text{NH}_4)_2\text{SO}_4$ fraction (30mg)	$7\alpha$ hydroxy-cholesterol	8.0
none	$7\alpha$ , $7\beta$ , hydroxy-cholesterol, 7-keto cholesterol	not determined

The effect of  $\beta$  mercaptoethylamine and a 40-80% ammonium sulphate fraction of rat liver 100,000g supernatant on cholesterol  $7\alpha$ hydroxylase from liver microsomes

Table 8.5

corresponding to the  $7\alpha$ -hydroxycholesterol was expressed as a ratio of the total radioactivity recovered. Table 8.1 shows that rat liver 100,000 g supernatant can stimulate the conversion of radioactive cholesterol to radioactive  $7\alpha$ -hydroxycholesterol and that this stimulation cannot be supported by the supernatant alone or by an equivalent amount of bovine serum albumin. Thus the results are comparable with Spence and Gaylor (13) who used a similar ratio of supernatant protein to microsomal protein.

That a protein present in 100,000 g supernatant was involved in this stimulation was confirmed by treatment of the unfractionated supernatant (90 mg) with trypsin. Table 8.2 shows that trypsin abolished the stimulatory activity of the 100,000 g supernatant implying that the stimulation was protein mediated.

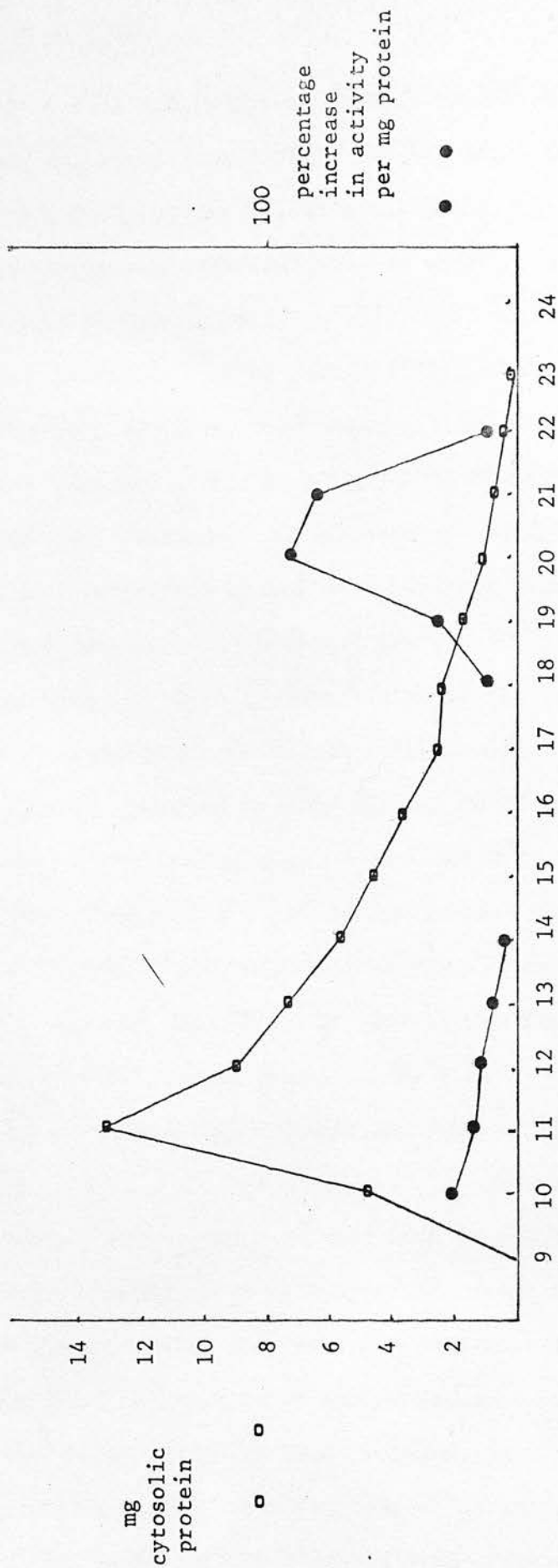
### 8.3 Preparation of an ammonium sulphate extract of 100,000 g supernatant

The active cytosolic protein was collected by salt precipitation of the 100,000 g supernatant. Solid ammonium sulphate was added to cytosol to 40% saturation over one hour with stirring and the pH was maintained at 7.4 with solid Tris base. The supernatant and salt precipitated protein were then separated by centrifugation for 15 min at 17,000 g. The pellet was discarded and the supernatant was brought to 80% saturation with solid ammonium sulphate with stirring as above. The precipitate was collected by centrifugation, dissolved in 50 mM phosphate buffer (pH 7.4) and dialysed overnight at 4°C. The protein was then dialysed against water for 3 hours and lyophilised. The recovery of 100,000 g protein in the 40-80 ammonium sulphate fraction varied between 25 and 38%. Upon addition of various amounts of the crude protein thus obtained, the activity of cholesterol  $7\alpha$ -hydroxylase

was increased over control levels (Table 8.3). It should be noted that preincubation of radioactive cholesterol with crude protein prior to addition to the enzyme did not improve conversion to product. Other workers (13) have shown that the cytosolic proteins could be further purified by heat treatment. Grabowski et al. (177) heated 30 mg supernatant protein/ml of solution in a boiling water bath until the temperature of the solution reached 60°C (approximately 2 minutes) and found that although 75% of the protein was denatured, the remaining 25% could enhance 12 $\alpha$ -hydroxylation of 7 $\alpha$ -hydroxy-4-cholesten-3-one. A similar procedure was repeated with the ammonium sulphate extract in order to assess if the protein that possessed cholesterol 7 $\alpha$ -hydroxylase stimulated activity was heat labile or heat stable. Heat treatment of the lyophilised ammonium sulphate fractionated cytosol at 80°C for 2 or 5 minutes resulted in the denaturation of 80-85 per cent of the protein, but Table 8.4 shows that the cytosolic protein may be partially heat stable. The preparation of Spence and Gaylor (13) that enhanced cholesterol 7 $\alpha$ -hydroxylase was seen to be heat labile under these conditions whereas the proteins that stimulated methyl sterol oxidase and HMG-CoA reductase were unaffected.

The effect of heat treatment on a protein which does not possess any innate biological activity is difficult to quantify. As the heat treatment denatures 80% of the protein but does not result in an increase in the stimulatory activity of the remaining 20%, the heat treatment at best is not improving the stimulatory activity of the cytosolic protein. The criteria for purification of such a protein must be based on the minimum amount that can cause maximum stimulation of the enzyme. Thus the apparent failure of a heat step may actually be due to the addition of

Fig. 8.1



Sephadex G75 gel filtration of cytosolic protein. Recovery of protein > 90%.



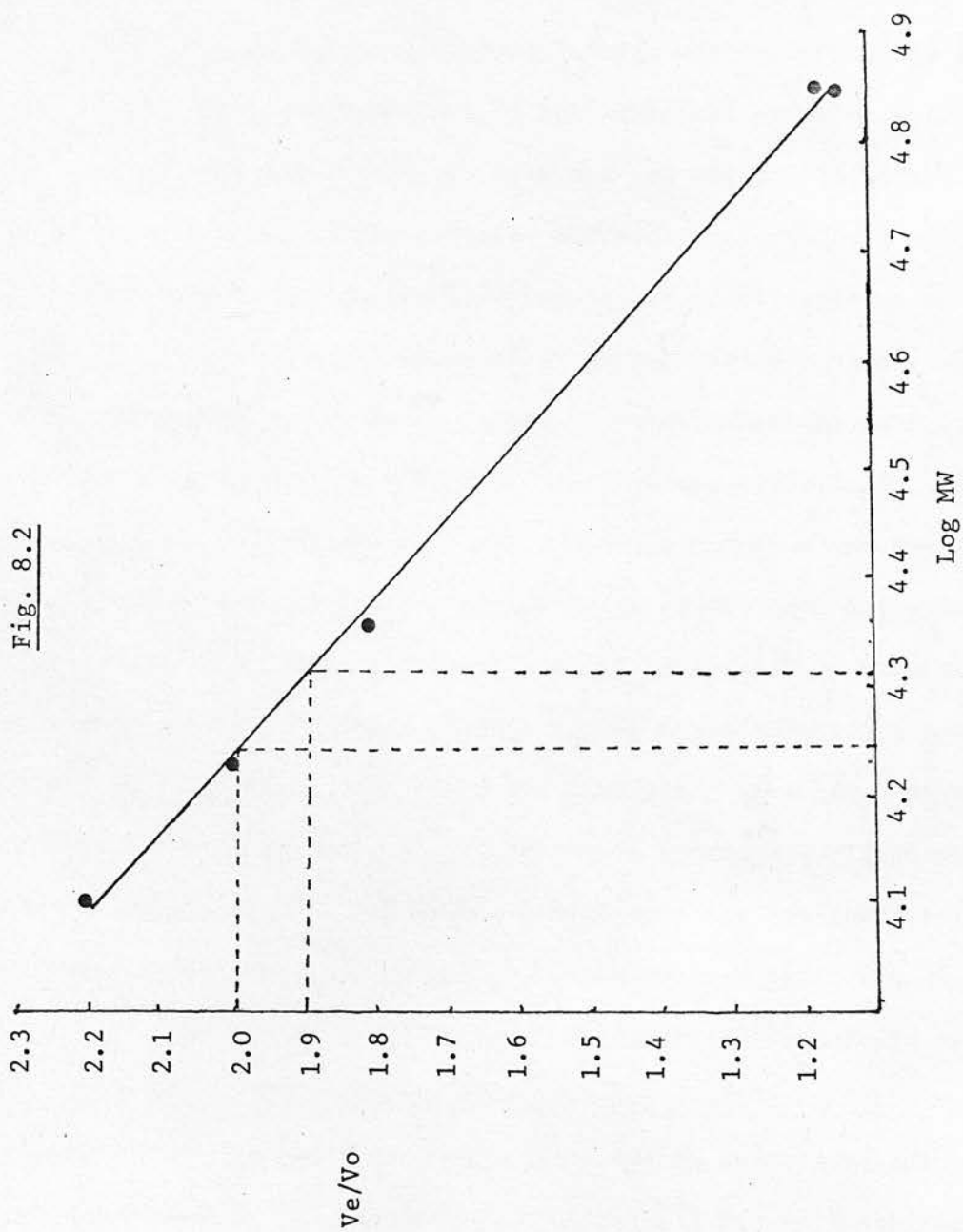
excess cytosolic protein. However this can be discounted in this case as the heated supernatant does not stimulate cholesterol 7 $\alpha$ -hydroxylase to the same extent as an equivalent amount of unheated cytosolic protein.

#### 8.4 Comparison of this cytosolic protein fraction with that of Scholan (180)

As a cytosolic factor had been implicated by Scholan in assisting metabolism of the radioactive cholesterol, its properties were compared with the cytosol protein fraction prepared by ammonium sulphate fractionation of the 100,000 g supernatant. The factor of Scholan was isolated from the supernatant of 100,000 g supernatant that had been boiled for 10 minutes at 95°C. It was non-dialysable and seemed to be acting to prevent auto-oxidation of the radioactive cholesterol in a similar way to the thiol  $\beta$ -mercaptoethylamine. Table 8.5 shows that the crude, heat labile, dialysable protein only partially acts as an anti-oxidant. Furthermore, a combination of a fraction containing the heat labile factors and  $\beta$ -mercaptoethylamine could stimulate the production of 7 $\alpha$ -hydroxycholesterol beyond the levels observed in the presence of  $\beta$ -mercaptoethylamine alone, whereas the heat stable fraction of Scholan could not. Although the crude heat labile protein fraction is partially preventing autoxidation, only in the presence of an anti-oxidant are its stimulating properties fully realised. Thus the SF described by Scholan and the cytosolic protein fraction seem to be playing different roles in cholesterol metabolism.

#### 8.4 Fractionation of the crude cytosolic protein by gel filtration

The properties of the crude supernatant protein were further investigated by gel filtration on a Sephadex G75 column prepared in 20 mM phosphate buffer pH 7.4. Gel filtration is common to all the



Molecular weight determination by use of a calibrated Sephadex G75 column.

reported purifications of cytosolic proteins and in combination with the heat treatment was seen to be the most effective in the purification scheme of Gaylor and Delwiche (181).

Various amounts of ammonium sulphate fractionated cytosol (50-300 mg protein) were applied to a 2 x 51 cm Sephadex G75 column in 20 mM phosphate buffer pH 7.4 (5 ml), and the eluent was collected in 5 ml aliquots. Figure 8.1 shows the elution profile of the cytosolic protein and the percentage increase in activity (as compared to the control values) when each fraction was added to a standard incubation mixture. Stimulatory activity was noted in fractions 10-12 and 19-21. When the increase in activity was expressed per milligram of the cytosolic protein added, maximal stimulation was associated with fractions 19-21. This pattern was observed over five independent estimations. If the protein eluting between 85-110 ml was concentrated and reappplied to the same column the elution position was unaltered. This implied that the void volume fraction was not an aggregate formed as a result of ionic strength effects.

Figure 8.2 shows the calibration curve of cytochrome c, myoglobin, trypsin, and bovine serum albumin applied individually to the Sephadex G75 column. The void volume was fixed at 51-52 ml by blue dextran, and as the ratio of  $V_e/V_o$  for the fractions with high specific activity varied between 1.9 and 2.1, the molecular weight of the protein species was estimated at between 17,300 and 20,000. Measurement of the  $A_{280}/A_{260}$  for protein in fraction 10-12 and 19-21 gave a value of 0.68-0.79 indicative of the presence of pyridine nucleotides.

Upon the addition of varying amounts of protein obtained from concentrating fractions 10-12 and 19-21 (Table 8.6) the enzyme was

Fraction 10-12 cytosolic protein(mg)	% conversion	Fraction 19-21 cytosolic protein(mg)	% conversion
1.25	5.6 $\pm$ 0.1	0.75	5.1 $\pm$ 0.2
3.75	5.9 $\pm$ 0.2	1.5	6.0 $\pm$ 0.5
7.5	5.4 $\pm$ 0.2	3	5.7 $\pm$ 0.1
16	6.2 $\pm$ 0.2	0	3.8
24	6.8 $\pm$ 0.1		
0	3.8 $\pm$ 0.2		

The effect of the addition of varying amounts of protein isolated from the void volume fraction and the retained fractions from Sephadex G/5 on the cholesterol  $\gamma$ -hydroxylase present in a microsomal preparation containing 15mg protein.

Table 8.6

cytosolic protein(mg) (A)	% conversion	cytosolic protein(mg) (B)	% conversion
3	6.1	1.5	5.0
1.5	5.8	0.75	4.7
0.75	5.5	0.50	4.4
0.5	5.2	0.25	4.9
0.25	5.9	0	2.9
0	2.9		

The effect of DEAE cellulose on the ability of the retained fraction ( G75 ) to stimulate cholesterol  $\Delta$ hydroxylase. (A) represents the fraction that is eluted by 5mM Tris HCl pH7.4, while (B) is eluted by 5mM Tris HCl pH7.4 containing 0.154 potassium chloride.

Table 8.7

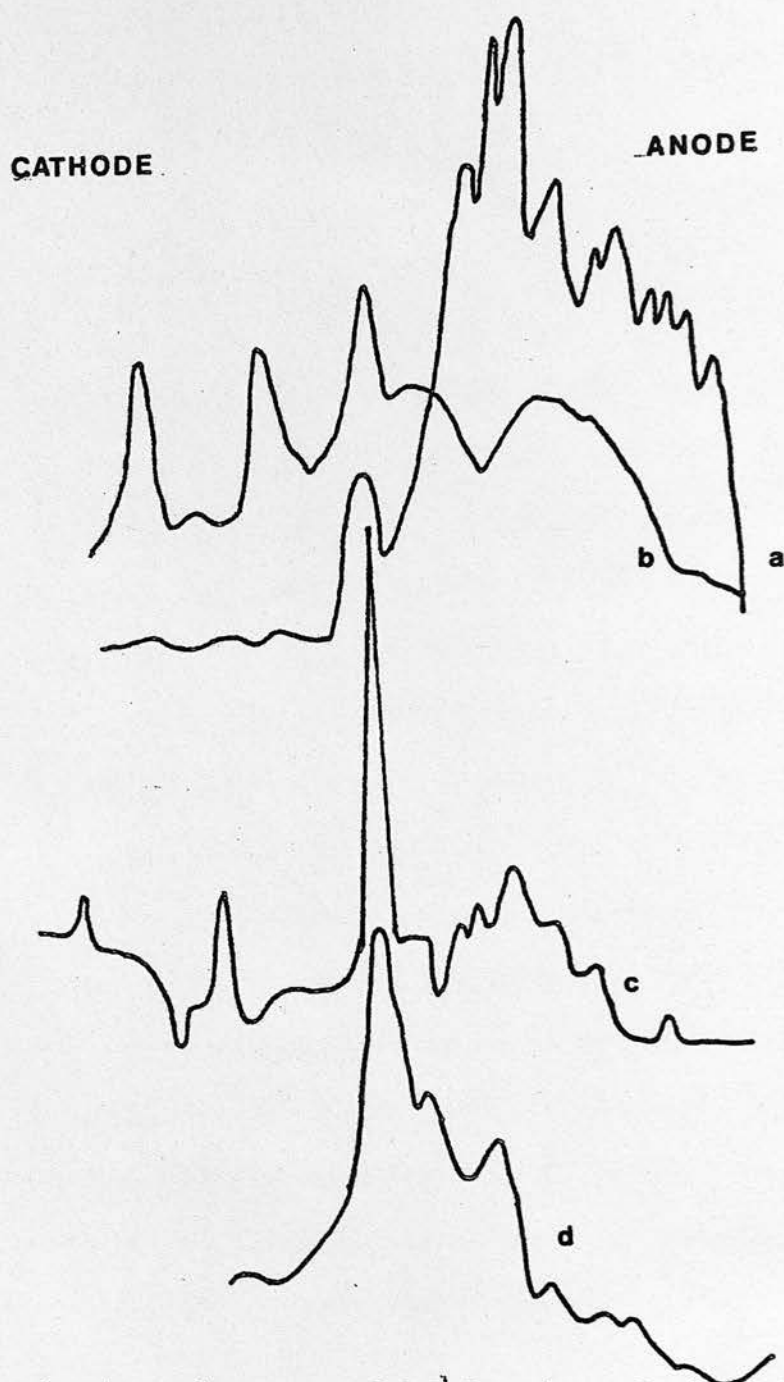
seen to be stimulated to near maximum values which never exceed 2 fold. When the percentage increase in the activity of the enzyme was calculated as a ratio of the cytosolic protein added, the protein in fraction 19-21 had been purified 38 fold by Sephadex treatment.

#### 8.6 Ion exchange chromatography of cytosolic proteins

The protein in fraction 17-22 (30 ml) of the Sephadex G75 column was concentrated by ultracentrifugation on an Amicon PM 10 membrane. This fraction (approx. 3 ml) <sup>was</sup> dialysed against 5 mM Tris HCl pH 7.4 (1 litre) for 12 hours at 4°C and applied to a 1.5 x 23 cm DEAE cellulose column equilibrated in the same buffer. The column was then washed with 5 mM Tris HCl pH 7.4 until no more protein was eluted, followed by elution with 5 mM Tris HCl pH 7.4 containing 0.154 M KCl which eluted a further protein peak. Protein was measured in 1 ml fractions and the two protein peaks were each pooled and concentrated by ultrafiltration using a PM 10 membrane in an Amicon ultrafiltration cell. The recovery of protein from the DEAE cellulose column varied between 50 and 60% (average of three estimations) and 70% of the recovered protein was associated with the first protein peak which was eluted from the DEAE cellulose by 5 mM Tris HCl pH 7.4. Although stimulatory activity seemed to be associated with both protein fragments, the more active protein was found in the first peak (Table 8.7).

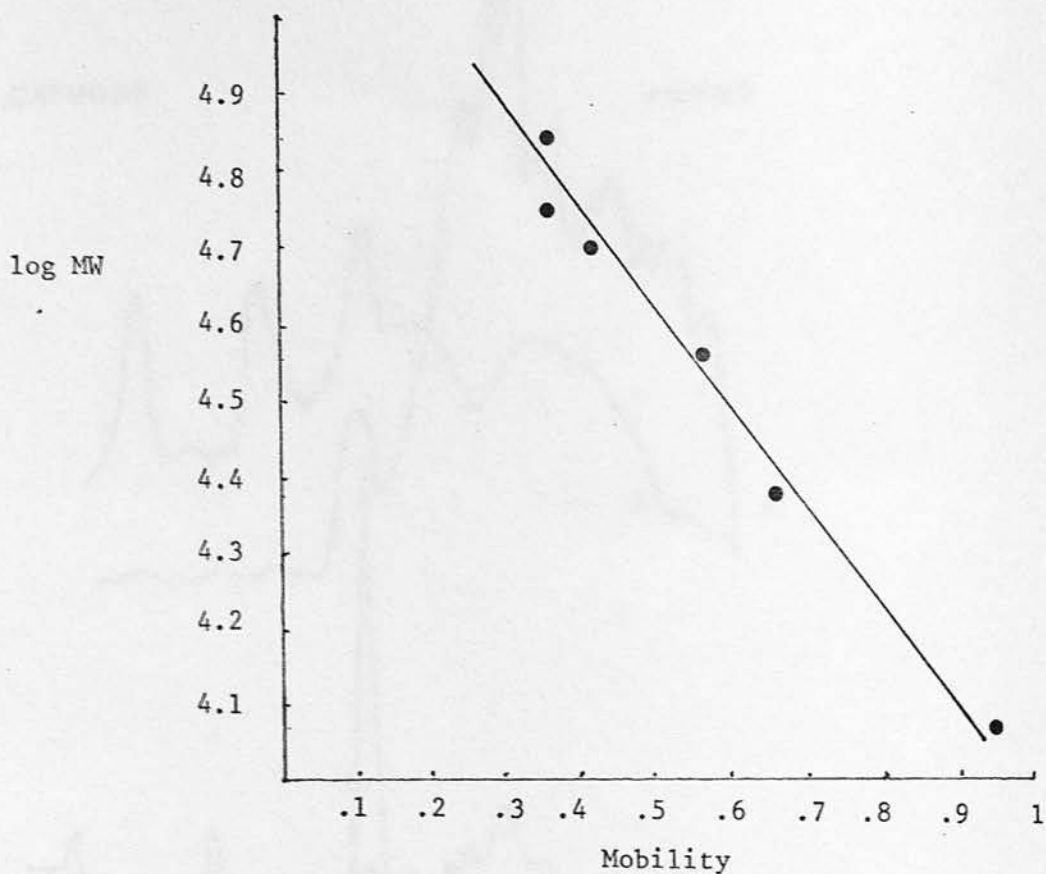
Ion exchange chromatography thus seems to further sub-fractionate and purify the cytosolic proteins that are associated with stimulation of cholesterol 7 $\alpha$ -hydroxylase. When activity is expressed as a percentage increase of product formed per mg of cytosolic protein the ion exchange chromatography step produces two fractions which are 415 and 275 fold purified





Relative intensity scans of S.D.S. polyacrylamide gels containing (a) fraction 10-12 from Sephadex G75 column (b) fraction 19-21 from Sephadex G75 (c) DEAE cellulose eluted with 5 mM Tris HCl pH 7.4 (d) DEAE cellulose eluted with 5 mM Tris HCl containing 0.154 M KCl pH 7.4

Fig. 8.3



S.D.S. polyacrylamide gel electrophoresis estimation of the molecular weight of the protein retained on Sephadex G75 by comparison with the mobilities of standard proteins. Standard proteins used, B.S.A. MW 68,000, cytochrome c MW 11,7000,  $\gamma$  globulin MW 50,600 and 23,500, glyceraldehyde 3P dehydrogenase MW 35,000, catalase MW 60,000 and ovalbumin MW 43,600.

Fig. 8.4

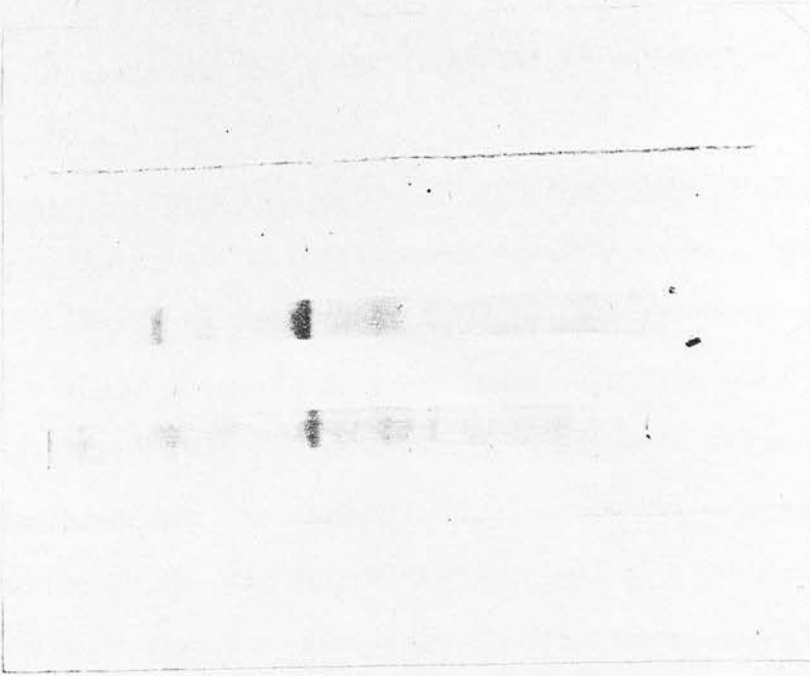
respectively as compared to the value for unfractionated supernatant.

#### 8.7 Sodium dodecyl sulphate-polyacrylamide disc gel electrophoresis

Each sample to be analysed was dialysed at room temperature for 12 hours against 100 volumes of 10 mM sodium phosphate buffer pH 7.0 containing 0.1% SDS and 0.1% mercaptoethanol in order to ensure that the sample composition would not interfere with the electrophoresis. The protein was then denatured by boiling in 0.01 M sodium phosphate buffer pH 7.0, 1% in SDS and 1% in mercaptoethanol for 3 minutes. A typical solution for application to the gel contained 5  $\mu$ l of tracking dye, 1 drop of glycerol, 5  $\mu$ l of mercaptoethanol, 10  $\mu$ l of protein, (0.6 mg/ml), and 50  $\mu$ l of dialysis buffer. Gel electrophoresis of the protein preparations was done in 0.1% sodium dodecyl sulphate (SDS) on 10% acrylamide essentially as described by Weber and Osborn (60).

Figure 8.3a-d shows relative density scans for each stage of purification. The Sephadex G75 treatment separates the proteins of the ammonium sulphate extract by molecular size. Analysis of the pooled fractions formed after gel filtration revealed that the material in fraction 10-12 consists of at least 20 protein bands, whereas apart from a lightly stained area adjacent to the origin of the gel which does not contain any visible bands, fraction 19-21 contained 5 protein bands, one of which migrated with the tracker dye. A similar analysis of the pooled fractions formed after ion exchange chromatography showed the following

(a) in the case of the protein which eluted in the equilibrating buffer the band with a relative mobility of 0.73 has become the most prominent of the bands and



Photograph of polyacrylamide gel electrophoresis (0.1% SDS) of cytosolic protein partially purified by ammonium sulphate, gel filtration, and ion exchange chromatography. Right hand side of photograph represents the anode.

Fig. 8.5

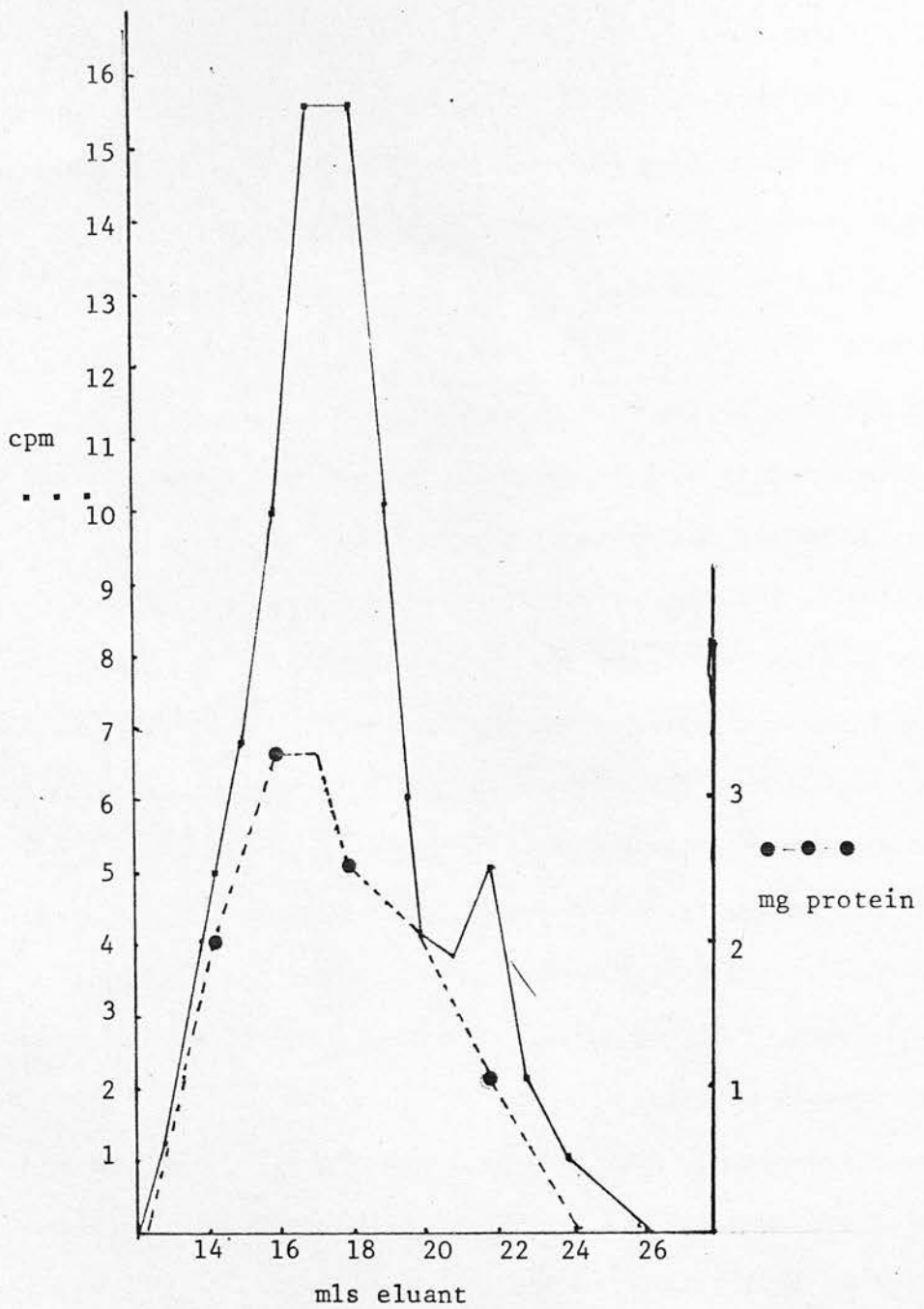


(b) that a less well defined species, with a similar relative mobility was present in the fraction eluted with equilibration buffer containing 0.154 M KCl. The relative mobilities of the main protein species observed are detailed in Table 8.8. It should be noted that the band with a mobility of 0.73 is present in all the fractions that stimulate cholesterol 7 $\alpha$ -hydroxylase.

An estimate of the molecular weight of the material with a relative mobility of 0.73 which constitutes the major protein band in the pooled ion exchange fractions was estimated by polyacrylamide gel electrophoresis by comparison with standard proteins of known molecular weight (Figure 8.4). This procedure gave the protein a molecular weight of 20,000-23,000 which was slightly higher than that determined by estimating the approximate elution position of a stimulating activity on a calibrated Sephadex G75 column (17,000-20,000). The differences may in part be explained by the inaccuracies in measurement of the column elution volumes. Figure 8.5 shows a photograph of the partially purified cytosol protein.

The purification techniques used this far have been partially successful and have yielded a preparation which consists of one major band MW 22,000 and five minor bands and whose activity is 400 fold greater than the 40-80%  $\text{NH}_4\text{SO}_4$  fraction from 100,000 g supernatant.

In addition molecular weight determination by SDS polyacrylamide electrophoresis is unaffected by the molecular dimensions of the protein, whereas molecular weight determinations by gel filtration are based on the Stokes radius of the proteins. Although further purification of the protein preparation will be



Elution profile of an incubation of  
radioactive cholesterol with cytosolic  
protein, partially purified by Sephadex G75  
gel filtration, from Sephadex G25.

Fig 8.6.



required before the precise identity of the stimulatory protein can be determined the 22,000 MW band is the major protein present in a stimulatory fraction and may be the protein responsible for the stimulation.

#### 8.8 Investigation of the possible method of action of the cytosolic protein

As the previously described cytosolic proteins have been designated as cholesterol binding protein (182), sterol carrier protein (174), or squalene binding protein (173), it was decided to investigate if the partially purified preparation obtained after Sephadex G75 chromatography could bind cholesterol. Fractions 17-22 (30 ml) from the Sephadex G75 column were concentrated to 3 ml in an Amicon filtration cell fitted with a PM 10 membrane. This concentrated protein (10 mg/ml) was then incubated with 60 nmole [ $^{14}\text{C}$ ] cholesterol at 37°C for 1 hour and applied to a 2 x 10.5 cm column of Sephadex G25 prepared in 20 mM phosphate buffer. Measuring the protein and radioactivity in the eluant showed that 40% of the added radioactivity is associated with a single void volume protein peak. The value is comparable with the binding achieved by the cytosol protein isolated by Grabowski et al. (177) which stimulates microsomal 12 $\alpha$ -hydroxylase. The stimulatory activity was also seen to be associated with the single void volume protein peak (Figure 8.6). When the radioactive cholesterol was applied to the column in the absence of the supernatant fraction, it could not be eluted from the Sephadex.

In an attempt to gain some extra information regarding the interaction of the cholesterol with the cytosolic protein a slightly different system for measuring the interaction between

Fractionation method	Mobility
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (40-80%) (A)	0.279 many bands, 0.460 (minor), 0.489 (minor), 0.558 (major), 0.71 (major).
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (40-80%) (B) Sephadex G75 fraction 10-12	20 bands. 0.468 (major) 0.495 (major), 0.588 (major), 0.669 (minor) 0.749 (minor)
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (40-80%) (C) Sephadex G75 fraction 19-21	0.586 (major), 0.778 (major), 0.836 (major) are the main proteins.
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (40-80%) (D) Sephadex G75 fraction 17-22, DEAE cellulose eluted with 5mM Tris HCl pH 7.4	0.43 (minor), 0.46 (minor), 0.47 (minor) 0.53 (minor), 0.73 (major), 0.82 (minor)
(E) as (D) except that the 5mM Tris HCl pH 7.4 contains 0.154M KCl	0.509 (minor), 0.647 (minor), 0.715 (minor)

A description of the S.D.S. polyacrylamide gels containing partially purified cytosolic protein

Table 8.8

Properties	Dempsey	Scallen	Ericksson
No. of proteins	1	3	1
Designation	SCP	SCP <sub>1</sub> , SCP <sub>2</sub> , SCP <sub>3</sub>	CBP
Function	conversion of (a) $\Delta^7$ cholesterol to 7-dehydrocholesterol. (b) 7-dehydrocholesterol to cholesterol (c) sterol to bile acid	SCP <sub>1</sub> -squalene <sub>1</sub> to lanosterol SCP <sub>2</sub> -4,4-dimethyl- $\Delta^8$ cholesterol to C <sub>27</sub> sterols SCP <sub>3</sub> - similar <sub>3</sub> to SCP (a,b)	cholesterol binding
Heat stability	yes (see note)	SCP <sub>1</sub> NO SCP <sub>2</sub> NO SCP <sub>3</sub> partial	not determined
Molecular weight 1% S.D.S	16,000	SCP <sub>1</sub> 50,000	26,500
gel filtration	16,000	-----	49,000
Electrophoretic mobility	0.77	SCP <sub>1</sub> 0.58	0.61
Purification	720 fold	SCP <sub>1</sub> 575 fold SCP <sub>3</sub> 112 fold	100 fold
Bound cholesterol	0	0	0.1-1.0 n mole/ n mole protein

Table 8.9  
Comparison of 3 cytosolic proteins

Table 8.9 shows a comparison between the best characterised cytosolic proteins.

Note- Astruc et al. (169) have suggested that boiled 100,000g supernatant cannot activate the microsomal conversion of squalene to cholesterol. Thus the reported heat stability of the Dempsey preparation must remain in doubt.

cholesterol and protein was adopted., The procedure used was that originally developed by Mester (183) for the determination of the binding characteristics of uterine cytosol proteins with different affinities for oestradiol and involved a 4°C overnight incubation of the cytosol with radioactive cholesterol to allow equilibration of the cholesterol and the cytosolic protein to occur. After the incubation period the unbound radioactivity was removed by treatment with a dextran-charcoal suspension. This assay is based on the fact that the charcoal adsorbs free but not protein-bound sterol. Provided the charcoal containing reaction mixture is vigorously but briefly agitated and the charcoal immediately centrifuged, a single determination of the radioactivity is sufficient to determine the percentage of added sterol that is bound to the cytosol protein. The degree of specificity of the binding was determined by comparison with boiled cytosol which measures non-specific binding. These procedures were repeated exactly using native and heat denatured cytosolic protein from fraction 17-21 of the Sephadex G75 gel filtration step, but results were obtained which indicated that there was no binding of the radioactive cholesterol to the cytosolic fraction tested. A possible explanation of this negative result could be that the binding of cholesterol to this protein is of such low affinity that the charcoal can remove bound cholesterol. This might be expected if the function of the protein was to transport the cholesterol from its site of synthesis to its point of metabolism.

Each method of measurement of binding gave different results which are probably attributable to the experimental limitation of each technique. An explanation of the ability of the protein to

bind cholesterol may be that the 37°C incubation aggregated the cytosolic proteins and created a molecular species capable of binding the cholesterol. This would agree with the findings of Ritter and Dempsey (174) that only the aggregated protein can bind cholesterol while the monomer sterol carrier protein will not. In their experience when a fraction from a gel filtration column containing apo SCP was incubated with a labelled cholesterol precursor and subjected to gel filtration, a radioactive protein with a higher molecular weight appeared in the void volume of the column. This observation is consistent with the results of gel filtration of the incubation mixture containing cytosolic protein and cholesterol that are presented in Figure 8.6.

Thus while the inclusion of cytosolic protein was observed to increase the production of 7 $\alpha$ -hydroxycholesterol from cholesterol its means of action must remain a matter of conjecture.

#### 8.9 Comparison of properties of reported cytosolic protein

In recent years the properties of some of the cytosolic proteins have been investigated to determine their structure and functional role. Ritter and Dempsey (174) have isolated a protein (purified to homogeneity as indicated by SDS) which has sterol carrier protein activity in the conversion of  $\Delta^7$ -cholesterol to 7-dehydrocholesterol and of 7-dehydrocholesterol to cholesterol. This protein was obtained by heat treatment of rat liver 100,000 g supernatant (1000, 5 minutes) followed by ammonium sulphate fractionation (40-80%) and passage through a Sephadex G75 column prepared and eluted with 0.1 M phosphate buffer pH 7.4. Table 8.13 shows some of these properties and in addition (a) only an aggregated form of the protein with a molecular weight in excess of 150,000 will bind substrate and



(b) only a protein-bound cholesterol will act as substrate for the enzyme. In addition phospholipid facilitated formation of the protein aggregate and apo HDL could replace SCP.

Scallen et al. (178) have obtained and partially purified three cytosolic proteins designated SCP<sub>1</sub>, SCP<sub>2</sub> and SCP<sub>3</sub>, respectively, which function in the conversion of squalene to cholesterol. Table 8.8 shows some of the main properties of the proteins and indicates that SCP<sub>3</sub> resembles SCP of Dempsey. The molecular weight of SCP<sub>1</sub> has been estimated to be 28,000 (182) or 13,000 depending on the conditions employed.

Both of these protein preparations have an enzyme activating role as their major cellular function. In the recent paper by Erickson et al. (187) a protein was described which had no stimulating function and was termed a cholesterol binding protein (CBP). The protein was initially tagged with cholesterol by intraperitoneal injection of radioactive cholesterol. The protein associated with the administered radioactivity was purified to homogeneity from rat liver 100,000 g supernatant by ammonium sulphate fractionation, gel filtration and ion exchange chromatography. Unlike the preparations of Dempsey and Scallen the purified protein contained cholesterol (Table 8.9) and differed in its immunochemical reactivity and could not be replaced by apo HDL.

The results of double immunodiffusion experiments with anti CBP antiserum and washed mitochondria, microsomes, rat liver nuclei and several other rat cytosols including kidney, spleen and adrenal imply that cholesterol binding proteins are found in many body tissues.

## Summary

In this chapter the involvement of a cytosolic protein in the microsomal conversion of cholesterol to 7 $\alpha$ -hydroxy-cholesterol has been demonstrated. This protein can be obtained by ammonium sulphate fractionation of the 100,000 g supernatant and is partially heat stable. The material possessing the stimulatory activity can be further subfractionated by gel filtration into two fractions

- (a) a fraction found at the void volume of Sephadex G75 equilibrated in 20 mM phosphate buffer pH 7.4 and
- (b) a fraction eluting at approximately twice the void volume.

Reapplication of fraction (b) to the same column did not alter the elution position of the fraction, implying that the void volume fraction was not a simple aggregate formed by ionic interaction of the monomers.

Both fractions have an  $A_{280}/A_{260}$  of 0.68 to 0.79 which is indicative of bound pyridine nucleotide. Ion exchange chromatography of the retained fractions on DEAE cellulose prepared in 5 mM Tris HCl pH 7.4 and eluted with 5 mM Tris HCl pH 7.4 and the same buffer containing 0.154 M KCl gave a further two protein fractions both of which stimulated enzymic activity. The maximum stimulating activity was associated with the peak eluted by 5 mM Tris HCl pH 7.4 and SDS polyacrylamide gel electrophoresis of this protein showed it to consist of one major band with an  $R_f$  of 0.73 (estimated molecular weight 19,000-23,000) and at least 5 minor bands. The SDS results probably provide a better estimate of the molecular weight of the protein than the results with the calibrated Sephadex G75 column as the molecular weight standards were of higher quality and the protein was purer.

These preliminary studies, although they are far from complete, suggest the existence of a rat liver cytosol<sup>protein</sup> capable of stimulating the activity of cholesterol 7 $\alpha$ -hydroxylase. The protein seems to resemble the CBP of Erickson more closely than the others due to the fact that the ion exchange purified material contained 0.5 mole cholesterol/mole of cytosol protein whereas the more purified preparations of SCP did not. In addition the molecular weight of the 7 $\alpha$ -stimulating protein by SDS gel electrophoresis was more akin to that of CBP than the others described in the literature. However the similarities of the  $A_{280}/A_{260}$  ratio between SCP and crude 7 $\alpha$ -stimulating protein indicate that they may have some properties in common. The presence of the endogenous cholesterol in the crude preparation of cytosol protein may in part explain the failure of the radioactive cholesterol to bind to the protein but its presence in an inhomogeneous preparation cannot be taken to indicate conclusively that it was acting as a cholesterol transport protein.

Whether the protein isolated in 100,000 g supernatant is a microsomal peripheral protein acting as a regulator of the movement of cholesterol from the general microsomal pool to a specific pool which provides substrate for the enzyme or is a cytosol protein acting as a means of supply of extra microsomal cholesterol is a question which cannot be answered at present.

Model for the distribution of cholesterol in rat liver microsomes.

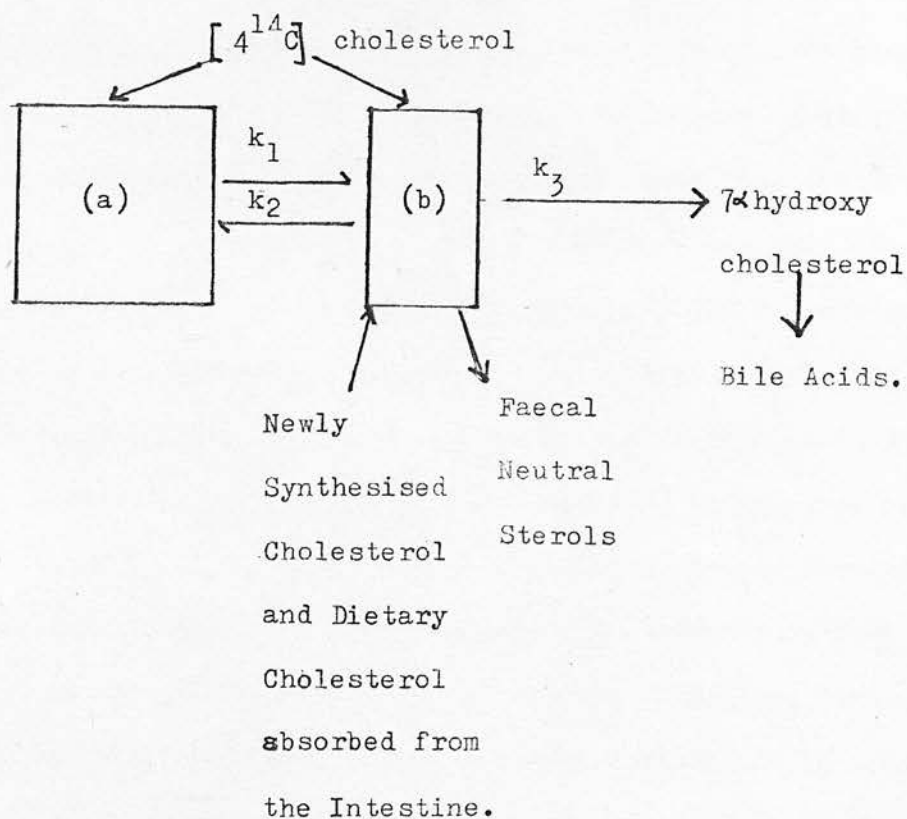


Fig. 9.1

## CONCLUSION

In mammalian cells cholesterol is the major sterol present. The structure of cholesterol with its rigid ring system, its secondary hydroxyl group at  $C_3$  and a branched chain at  $C_{17}$ , is thought to determine its biochemical properties. In previous studies, the cholesterol nucleus has been the main focus of attention with the side chain of cholesterol only being mentioned in passing.

The object of the work described in this thesis was two-fold

(a) definition of the precise dimension of the side chain at  $C_{17}$  necessary to optimise the biochemical properties of the cholesterol molecule.

(b) further investigation of the two main cholesterol systems of the adrenal and the liver.

In the liver cholesterol is present as a membrane structural component and as a substrate for certain microsomal oxidising enzymes. The relative proportions of metabolically restrained cholesterol in liver microsomes are unknown. A possible model for cholesterol compartmentation in liver microsomes is shown in figure 9.1.

Pool (a) represents metabolically inactive (structural) cholesterol which equilibrates at a slow rate with pool (b) which is assumed to be metabolically active. The cholesterol in pool (b) can be converted to bile acids or excreted as faecal neutral sterols and can be replenished by de novo hepatic synthesis and by cholesterol absorption from the gut.

The radiotracer assay of cholesterol  $7\alpha$  hydrolase assumes that exogenous cholesterol equilibrates with endogenous cholesterol in pools (a) and (b). The similar results obtained for the GLC and radioactive tracer assays (Table 6.7) indicate that equilibration is occurring.

The association of substrate cholesterol with cholesterol 7 $\alpha$ -hydroxylase might be a control step in the production of cholesterol 7 $\alpha$ -hydroxycholesterol although it has not been possible to demonstrate a specific in vitro interaction of cholesterol with the enzyme (184). Alternatively the activity of cholesterol 7 $\alpha$ -hydroxylase could be enhanced by increasing the supply of substrate for the enzyme, by altering the relative preparations of microsomal cholesterol in pools (a) and (b). This may be achieved by cholesterol feeding which is known to stimulate cholesterol 7 $\alpha$ -hydroxylase (9), or by the existence of a specific cholesterol transport mechanism from pool (a) to pool (b).

The protein described in chapter 8 enhances the existing activity of cholesterol 7 $\alpha$ -hydroxylase but is not a prerequisite for enzyme activity. Thus it is more likely to be providing substrate than forming an integral part of the active site. The existence of the protein might also explain the observation that the cholestyramine mediated increase in activity was removed by treatment with actinomycin D (2,3). Evidence of a direct relationship between the synthesis of a protein which does not possess any innate catalytic activity and the activity of cholesterol 7 $\alpha$ -hydroxylase would be difficult to establish.

As HMG CoA reductase and cholesterol 7 $\alpha$ -hydroxylase are both thought to be situated in the smooth endoplasmic reticulum (185) and newly synthesised cholesterol is believed to be the preferred substrate for cholesterol 7 $\alpha$ -hydroxylase (164) then the role of the protein might be to redistribute the microsomal cholesterol rather than to bring cholesterol to the microsomes. Furthermore the location of both enzymes on the same membrane would suggest that the "cytosolic" protein could be a microsomal peripheral protein that has been removed from the microsomes during the isolation procedure.



Cholesterol 7 $\alpha$ -hydroxylase is thought to be cytochrome P450 linked as demonstrated by the inhibition of the enzyme by carbon monoxide and by reversal of the inhibition with monochromatic light at 450 nm (129,186). There is no direct correlation between the level of hepatic cytochrome P450 and cholesterol 7 $\alpha$ -hydroxylase. There may be a cytochrome P450 specifically associated with cholesterol 7 $\alpha$ -hydroxylase which constitutes such a small proportion of the total cytochrome P450 that its induction following cholestyramine treatment is not observed. The results of chapter 6 involving drug pre-treatment of animals and comparison of the metabolism of pregn-5-en-3 $\beta$ -ol ( $C_{21}$ ), and 23-24-bisnor-5-cholen-3 $\beta$ -ol ( $C_{22}$ ) with cholesterol showed that Pb pretreatment increased hepatic cytochrome P450 levels and enhanced ethylmorphine demethylase activity and the metabolism of  $C_{21}$  and  $C_{22}$  to many products. The activity of cholesterol 7 $\alpha$ -hydroxylase was unaffected and only gave one major product in the presence of  $\beta$ -mercaptoethylamine. Thus the metabolism of the short side chain analogues seemed to be more related to drug metabolism enzymes than to the cholesterol 7 $\alpha$ -hydroxylase system.

This relationship between the structure of the cholesterol analogue and its pattern of metabolism could be due to the existence of a hydrophobic cleft in the active site of cholesterol 7 $\alpha$ -hydroxylase. This cleft would be optimally adapted to fit the iso-octane side chain of cholesterol, but not sterols with longer or shorter side chains.

The side chain cleavage enzyme system of rat adrenal mitochondria is another cholesterol oxidising system that has been investigated with a cholesterol side chain analogues to determine if the side chain has a role to play in regulating the enzyme activity.

In a recent paper Arthur et al. (141) reported that sterols with the cholest-5-en-3 $\beta$ -ol ring system and saturated side chains of different lengths were converted to pregnenolone at rates similar to cholesterol. When the experiments were repeated using ACTH pretreated rats and  $\text{Ca}^{2+}$  in the incubation medium a different result was obtained which indicated that cholesterol itself was the optimal substrate for the adrenal side chain cleavage system. In the mitochondria the substrate cholesterol could come from the plasma lipoproteins or from lipid droplets present in the adrenal cell cytoplasm or from de novo synthesis. ACTH is known to act on the cells of the adrenal cortex to produce an almost instantaneous stimulation of steroidogenesis. The effects of calcium on side chain cleavage is not so well understood, although it has been shown to increase side chain cleavage activity in rat testes mitochondria (187).  $\text{Ca}^{2+}$  is also thought to bind to the phosphodiester groups of phospholipid. The affinity of  $\text{Ca}^{2+}$  for PC bilayers is such that at 5 mM approx. 1% of all phosphatidylcholine polar groups are likely to be bound. The cation bound conformation of the hydrophobic bilayer region may be different from the ion free state of the bilayer (188). However this interaction between phosphatidylcholine polar groups and  $\text{Ca}^{2+}$  is weak and does not change the packing density and the molecular motion of the hydrocarbon chains.

Phosphatidylcholine is zwitterionic over physiological pH range and the affinity of  $\text{Ca}^{2+}$  is much lower than that for negatively charged phospholipid such as phosphatidylserine and phosphatidylethanolamine. The lipid of the internal mitochondrial membrane which constitutes 25% of the membrane mass, contains 90% phospholipids composed of phosphatidylcholine, phosphatidylethanolamine and cardiolipin in

approximate molar ratios of 4:3:2 (189). Thus there are many sites where  $\text{Ca}^{2+}$  could bind to the phospholipid head groups.

The current evidence favours the concept that the availability of cholesterol to the enzyme regulates pregnenolone synthesis rather than alteration in the amount of enzyme present. This concept is consistent with other properties of membranes containing  $\text{Ca}^{2+}$ . Results of a study involving interacting spin labels show that  $\text{Ca}^{2+}$  may increase the extent of phase separation in phosphatidic acid-phosphatidylcholine membranes (190). This induced phase separation is caused by formation of crystalline patches of calcium ion bound phosphatidic acid, the phosphatidylcholine being squeezed from the areas of rigid lipids (69). The alteration in phase separation caused by  $\text{Ca}^{2+}$  could result in the displacement of cholesterol from one region to another, this second region being more accessible to the enzyme complex. The differential metabolism of the various cholesterol side chain analogues could then be explained in terms of their relative abilities to migrate in the mitochondrial membrane. That this property and not direct interaction with enzyme is the determining factor in pregnenolone production from the analogues is expected, as previously in the absence of  $\text{Ca}^{2+}$ , all the analogues had been shown to act as equivalent substrates for the side chain cleavage enzyme (141).

The possible dependence of translational mobility of the cholesterol side chain analogues on the side chain structure correlates with the reported necessity of an intact side chain for the optimal sterol-phospholipid interaction described in chapter 4. The precise role of the side chain in cholesterol phospholipid interactions was investigated using cholesterol analogues with altered side chains and a selection of physical techniques to monitor the interaction.

The epr studies described in chapter 4 showed that the various spin labels were reporting on different areas of the bilayer as judged by the differential removal of each spin label signal by ascorbate. The results of all the spin labelling experiments indicated that cholesterol with its iso-octane side chain was optimally adapted for maximal interaction with the surrounding phospholipid. That the cholesterol side chain and not the phospholipid fatty acyl chain was determining the extent of sterol phospholipid interaction was confirmed when alteration of the phospholipid species was seen to have no effect on the previously observed pattern of cholesterol phospholipid interaction (Fig. 4.15). In the case of the experiments with the 3NC spin label 30 mole % sterol had to be present to show the side chain effect, and in agreement with Engelman and Rothman (191) this was the proportion of cholesterol that required to be present to prevent the phospholipid phase transition from occurring.

The results of chapter 4 and the osmotic shrinking experiments outlined in chapter 5 using liposomes containing the cholesterol side chain analogues confirmed the correlation between spin labelling and osmotic shrinking experiments as observed by other groups (122). Furthermore they showed that the effect of cholesterol on both physical processes was closely linked with the cholesterol iso-octane side chain. When the interaction of the cholesterol analogues with phospholipid was assayed using a mixed monolayer the importance of the cholesterol side chain was not so apparent since apart from C<sub>19</sub> and C<sub>21</sub> the rest of the analogues caused the monolayer to condense to the same extent as cholesterol. This result could be explained by suggesting that cholesterol required to be incorporated into a bilayer structure of fixed dimensions before the properties of the side chain could be fully expressed. Further evidence for this

interpretation came from the report of Forge et al. (117) that cholesterol was capable of exerting a molecular control of membrane morphology in areas with a high radius of curvature.

The side chain of cholesterol is also important in determining the rate of exchange of liposomally bound cholesterol analogues as observed in chapter 7. Of the side chain analogues tested, cholesterol seemed to exchange most readily into microsomes. The figures obtained for the percentage of liposomal sterol available for exchange can only be used for comparison of the series of analogues and not as an absolute index of the amount of liposomal or microsomal cholesterol that is available for exchange. That this is the case was shown by the different results obtained for the amount of liposomal cholesterol that is available for exchange into the erythrocyte membrane. As the rate of exchange of cholesterol from a liposome is thought to be dependent on the efficiency <sup>of</sup> conversion of multilayered to single layered liposomes (151) comparison of different results is difficult.

The results of both the physical measurements and the enzyme assays reveal that the iso-octane side chain of cholesterol is playing a major role in determining the extent of the interaction between cholesterol and phospholipids and the specificity of cholesterol protein interactions. A possible future use for these side chain analogues could be to further investigate the role of cholesterol in several other natural membranes including liver microsomes, platelets and erythrocytes by replacing cholesterol with liposomally delivered cholesterol analogues. Erythrocyte fusion requires the creation of protein free, fluid areas of lipid which form the fusion site (192). Cholesterol is thought to be important in the formation of the protein free area. As the cholesterol side



chain is involved in the interaction of cholesterol with lipid and protein, the extent of phase separation and hence of cell fusion may be dependent on the interaction of the cholesterol side chain with protein or lipid. The techniques developed in this work should be adaptable to the study of this aspect of membrane organisation.

1. Shalvey, J., Bamber, R., and Mowbray, R.H. (1976) *J. Lipid Res.*, **17**, 329-333.
2. Einarsson, R., and Johannsen, C. (1969) *FEBS Lett.*, **1**, 218-222.
3. Krieger, R., Haver, R., Burdick, J., and Mowbray, R.H. (1970) *J. Lipid Res.*, **11**, 403-412.
4. Denigese, R., Einarsson, R., and Johannsen, C. (1967) *Eur. J. Biochem.*, **2**, 45-48.
5. Hume, R. (1978) *Biochem. Soc. Trans.*, **6**, (in press).
6. Boyd, R.S., Scholten, R.A., and Wilson, J.B. (1969) *Drugs Affecting Lipid Metabolism*, Plenum Press, 443-456.
7. Boyd, R.S., Brown, R.J.G., Hattersley, R.H., and Pickling, R.E. (1974) *Biochem. Biophys. Acta*, **337**, 174-182.
8. Pickling, R.E., and Denigese, R. (1975) *Eur. J. Biochem.*, **53**, 63-70.
9. Spagnol, A., Gell, R., and Gell, R. (1976) *Anal. Biochem.*, **71**, 430-436.
10. Spagnol, R., and Taylor, J.L. (1979) *J. Biol. Chem.*, **254**, 5032-5036.
11. Klapper, R., and Scholten, R. (1977) *FEBS Lett.*, **90**, 423-425.
12. Klapper, R., Hume, R., and Brown, R.E. (1977) *Proc. Acad. Sci. U.S.A.*, **74**, 2507-2511.
13. Gell, R. (1975) *Biochem. Biophys. Acta*, **395**, 12-17.
14. Pickling, R.E., and La Penna, A.M. (1971) *Biochem. Biophys. Acta*, **278**, 15-22.



1. Björkhem, I., and Danielsson, H. (1974) *Molecular and Cellular Biochem.*, 4, 79-95.
2. Brown, M.J.G., and Boyd, G.S. (1974) *Eur. J. Biochem.*, 44, 37-47.
3. Gielen, J., Van Cantfort, J., Robaye, B., and Renson, J. (1973) *Eur. J. Biochem.*, 55, 41-48.
4. Shefer, S., Hauser, S., and Mosbach, E.H. (1968) *J. Lipid Res.*, 9, 328-333.
5. Einarsson, K., and Johansson, G. (1969) *FEBS Lett.* 1, 219-222.
6. Shefer, S., Hauser, S., Berksky, I., and Mosbach, E.H. (1970) *J. Lipid Res.*, 11, 404-411.
7. Danielsson, H., Einarsson, K., and Johansson, G. (1967) *Eur. J. Biochem.*, 2, 44-49.
8. Hume, R. (1978) *Biochem. Soc. Trans.*, 6, (in press).
9. Boyd, G.S., Scholan, N.A., and Mitton, J.R. (1969) *Drugs Affecting Lipid Metabolism*, Plenum Press, 443-456.
10. Boyd, G.S., Brown, M.J.G., Hattersley, N.G., and Suckling, K.E. (1974) *Biochem. Biophys. Acta*, 337, 132-135.
11. Björkhem, I., and Danielsson, H. (1975) *Eur. J. Biochem.*, 53, 63-70.
12. Sanghvi, A., Galli Kienli, M., and Galli, G. (1978) *Anal. Biochem.*, 85, 430-436.
13. Spence, J.T., and Gaylor, J.L. (1977) *J. Biol. Chem.*, 252, 5852-5856.
14. Klappauf, E., and Schubert, D. (1977) *FEBS Lett.*, 80, 423-425.
15. Kandutsch, A.A., Chen, H.W., and Shrun, E.P. (1977) *Proc. Natl. Acad. Sci. U.S.*, 74, 2500-2503.
16. Bell, F.P. (1975) *Biochem. Biophys. Acta*, 398, 18-27.
17. Dorling, P.R., and Le Page, R.N. (1973) *Biochem. Biophys. Acta*, 318, 33-40.

18. Ashworth, L.A.E., and Green, C. (1966) *Science*, 151, 210-211.
19. Hsia, J.C., Schneider, H., and Smith, J.C.P. (1971) *Can. J. Biochem.*, 49, 614-622.
20. Stockton, G.W., Pohnasyek, C.F., Leitch, L.C., Tullock, A.P., and Smith, I.C.P. (1974) *Biochem. Biophys. Res. Commun.*, 60, 844-850.
21. Levine, Y.K., and Wilkins, M.H. (1971) *Nat. New. Biol.*, 230, 69-72.
22. Lambbrooke, B.D., Williams, R.M., and Chapman, D. (1968) *Biochem. Biophys. Acta*, 150, 333-340.
23. Shah, D.O., and <sup>c</sup>Shulman, J.H. (1967) *J. Lip. Res.*, 8, 215-222.
24. de Kruffyff, B., Demel, R.A., Slatboom, A.J., van Dunen, L.L.M., and Rosenthal, A.F. (1973) *Biochem. Biophys. Acta*, 307, 1-9.
25. Bangham, A.D., Standish, M.M., and Watkins, J.C. (1965) *J. Mol. Biol.*, 13, 253-264.
26. Jain, M.K., Toussaint, D.G., and Cordes, E.H. (1973) *J. Membrane Biol.*, 14, 1-16.
27. Demel, R.A., Bruckdorfer, K.R., and van Deenen, L.L.M. (1972) *Biochem. Biophys. Acta*, 255, 321-330.
28. Demel, R.A., Guerts van Kessel, W.S.M., and van Deenen, L.L.M. (1972) *Biochem. Biophys. Acta*, 266, 26-40.
29. de Kruffyff, B., Demel, R.A., and van Deenen, L.L.M. (1972) *Biochem. Biophys. Acta*, 255, 331-347.
30. Suckling, K.E., and Boyd, G.S. (1976) *Biochem. Biophys. Acta*, 436, 295-300.
31. Hubbell, W.L., and McConnell, H.M. (1971) *J. Am. Chem. Soc.*, 93, 314-326.
32. Oldfield, E., and Chapman, D. (1972) *FEBS Lett.*, 23, 285-297.

33. Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H.,  
and Smith, I.C.P. (1973) *Chem. Phys. Lipids*, 10, 11-27.
34. Lippert, J.L., and Peticolas, W.L. (1971) *Proc. Natl. Acad. Sci. U.S.*, 68, 1572-1576.
35. Lancee-Hermkens, A.M.W., and de Kruyff, B. (1977) *Biochem. Biophys. Acta*, 470, 141-151.
36. de Kruyff, B., van Dijck, P.W.M., Demel, R.A., Schuijft, A.,  
Brants, F., and van Deenen, L.L.M. (1974) *Biochem. Biophys. Acta*, 356, 1-7.
37. Demel, R.A., Jansen, J.W.C.M., van Dijck, P.W.M., and van Deenen, L.L.M. (1977) *Biochem. Biophys. Acta*, 465, 1-10.
38. Cullis, P.R., and de Kruyff, B. (1978) *Biochem. Biophys. Acta*, 507, 207-218.
39. Bergelson, L.K., and Barsukov, L.I. (1977) *Science*, 197, 224-230.
40. Gottlieb, M.H. (1977) *Biochem. Biophys. Acta*, 466, 422-428.
41. Huang, C.H., Sipe, J.D., Chow, S.T., and Martin, R.B. (1974) *Proc. Natl. Acad. Sci. U.S.*, 71, 359-362.
42. Darke, A., Finn, E.G., Flovk, A.G., and Phillips, M.C. (1972) *J. Mol. Biol.*, 63, 265-279.
43. Cullis, P.R., de Kruyff, B., and Richards, R.E. (1975) *Biochem. Biophys. Acta*, 426, 433-446.
44. Rothman, J.E., and Engelman, D.M. (1972) *Nature*, 237, 42-44.
45. Opella, S.J., Yesinowski, J.P., and Waugh, J.S. (1976) *Proc. Natl. Acad. Sci. U.S.*, 73, 3812-3815.
46. Kroon, P.A., Keinoshio, M., and Chan, S.I. (1975) *Nature*, 256, 582-584.
47. Yeagle, P.L., Hutton, W.C., Huang, C.H., and Martin, R.B. (1977) *Biochemistry*, 16, 4344-4349.
48. Brown, M.F., and Sulig, J. (1978) *Biochemistry*, 17, 381-384.
49. Franks, N.P. (1976) *J. Mol. Biol.*, 100, 345-358.

50. Schwarz, F.T., and Pattauf, F. (1977) *Biochemistry*, 16, 4335-4339.
51. Huang, C. (1977) *Lipids*, 12, 348-356.
52. Brockerhoff, H. (1974) *Lipids*, 9, 645-650.
53. Ernster, L., Siekevitz, P., and Palade, G.E. (1962) *J. Cell. Biol.*, 15, 541-562.
54. Vore, M., Hamilton, J.G., and Lu, A.Y.H. (1974) *Biochem. Biophys. Res. Commun.*, 56, 1038-1044.
55. Mitton, J.R., Scholan, N.S., and Boyd, G.S. (1971) *Eur. J. Biochem.*, 20, 569-579.
56. Folch, J., Lees, M., and Stanley, G.H.S. (1957) *J. Biol. Chem.*, 226, 497-509.
57. Trinder, P. (1969) *Ann. Clin. Biochem.*, 6, 24-27.
58. Zilversmit, D.B., and Davis, A.K. (1950) *J. Lab. Clin. Med.*, 35, 357-366.
59. McClare, C.W.F. (1971) *Anal. Biochem.*, 39, 527-530.
60. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.*, 244, 4406-4412.
61. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.*, 193, 265-275.
62. Layne, E. (1957) *Methods in Enzymology* (Colowick, S.P., and Kaplan, N.O. eds.), 3, 450-451.
63. Omura, T., and Sato, R. (1964) *J. Biol. Chem.*, 239, 2370-2378.
64. Huang, C.H. (1969) *Biochemistry*, 8, 344-351.
65. Pangborn, M.C. (1951) *J. Biol. Chem.*, 188, 471-476.
66. Kaetherer, T. personal communication.
67. Griffith, O.H., and Jost, P.C. (1976) *Spin Labelling. Theory and Applications* (Berliner, I.J.) p. 453-519.
68. Smith, I.C.P. (1971) *Chimia*, 25, 349-360.
69. Shimshick, E.J., and McConnell, H.M. (1973) *Biochem. Biophys. Res. Commun.*, 53, 446-451.

70. de Boer, Th.J., and Backer, H.J. (1954) *Rec. trav.*, 73, 229-235.
71. Fernholz, E. (1933) *J. Liebigs Ann. Chem.*, 507, 128-135.
72. Fieser, L.F., and Fieser, M. (1967) *Reagents for Organic Synthesis* vol. 1 p. 583. Wiley - New York.
73. Bharuca, K.R., Buckley, G.C., Ress, C.K., Rubin, L.J., and Zieyler, P. (1956) *Can. J. Chem.*, 34, 982-900.
74. Helferich, B., Speidel, P.E., and Toeldte, W. (1923) *Ber.*, 56, 766-770.
75. Greenhalgh, C.W., Henbest, H.B., and Jones, E.R.H. (1951) *J. Chem. Soc.*, 1190-1193.
76. Dasgupta, S.K., Crump, D.R., and Gut, M. (1974) *J. Org. Chem.*, 39, 1658-1662.
77. Collins, J.C., Hess, W.W., and Frank, F.J. (1968) *FEBS Lett.*, 30, 3363-3366.
78. Fried, J., and Edwards, J.A. (1972) *Organic Reactions in Steroid Biochemistry*. van Nostrand Reinhold - New York.
79. Petrov, V., and Stuart-Webb, I.A. (1956) *J. Chem. Soc.*, 4675-4677.
80. Malinow, M.R., McLaughlin, P., Papworth, L., and Kittingen, G.W. (1975) *Steroids*, 25, 663-672.
81. Johnson, D.B., and Lack, L. (1976) *J. Lipid Res.*, 17, 91-93.
82. Schink, G.O., Neumuller, O.A., and Eisfeld, W. (1959) *J. Liebigs Ann. Chem.*, 618, 202-210.
83. Hubbell, W.L., and McConnell, H.M. (1969) *Proc. Natl. Acad. Sci. U.S.*, 63, 16-22.
84. Hubbell, W.L., and McConnell, H.M. (1969) *Proc. Natl. Acad. Sci. U.S.*, 64, 20-27.
85. Oldfield, E., and Chapman, D. (1971) *Biochem. Biophys. Res. Commun.*, 43, 610-616.

86. Hemminga, M.A. (1975) *Chim. Phys. Lipids*, 14, 141-150.
87. Wu, S.H., and McConnell, H.M. (1974) *Biochemistry*, 14, 847-854.
88. Sackmann, E., and Träuble, H. (1972) *J. Am. Chem. Soc.*, 94, 4482-4491.
89. Demel, R.A., Bruckdufer, K.R., and van Deemen, L.L.M. (1972) *Biochem. Biophys. Acta*, 255, 311-320.
90. Cadenhead, D.A., Demchak, R.J., and Muller-Landau, F. (1972) *Ann. N.Y. Acad. Sci.*, 195, 218-223.
91. Tinow, J., Ghosh, D., and Keith, A.D. (1972) *Biochim. Biophys. Acta*, 274, 279-285.
92. Smith, I.C.P., and Butler, K.W. (1976) *Spin Labelling - Theory and Applications*, p. 411-453. Academic Press.
93. Seelig, J. (1976) *Spin Labelling - Theory and Applications*, p. 373-410. Academic Press.
94. Lapper, R.D., Paterson, S.J., and Smith, I.C.P. (1972) *Can. J. Biochem.*, 50, 969-981.
95. Butler, K.W., Smith, I.C.P., and Schneider, H. (1970) *Biochim. Biophys. Acta*, 337, 132-135.
96. Chapman, D. (1975) *Quarterly Rev. Biophysics*, 8, 185-235.
97. Rand, R.P., Chapman, D., and Larsson, K. (1975) *Biophys. J.*, 15, 1117-1124.
98. Levine, Y.K., Birdsall, N.J.M., Lee, A.G., and Metcalfe, J.C. (1972) *Biochemistry*, 11, 1416-1421.
99. Hing, H.J., and Sturtevant, J.M. (1972) *J. Biol. Chem.*, 247, 3697-3700.
100. Demel, R.A., and de Kruffy, B. (1976) *Biochim. Biophys. Acta*, 457, 109-132.
101. Marsh, D., Watts, A., and Knowles, P.F. (1977) *Biochem. Biophys. Acta*, 465, 500-514.



102. Linden, C.D., Keith, A.D., and Fox, C.F. (1973) *J. Supramolecular Structure*, 1, 523-534.
103. Schreier-Muccillo, S., Butler, K.W., and Smith, I.C.P. (1976) *Arch. Biochem. Biophys.*, 172, 1-11.
104. Norman, A.W., Demel, R.A., de Kruyff, B., Geurts van Kessel, W.S.M., and van Deenen, L.L.M. (1972) *Biochim. Biophys. Acta*, 290, 1-14.
105. Bittman, R., Chen, W.C., and Anderson, O.R. (1974) *Biochemistry*, 13, 1364-1373.
106. Hayden, D.A., and Taylor, J. (1963) *J. Theoret. Biol.*, 4, 281-296.
107. Bangham, A.D., and Horne, R.W. (1964) *J. Mol. Biol.*, 8, 660-668.
108. Bittman, R., and Blau, L. (1972) *Biochemistry*, 11, 4831-4838.
109. Bangham, A.D., de Gŭr, J., and Greville, G.D. (1967) *Chem. Phys. Lipids*, 1, 225-246.
110. Lieb, W.R., and Stein, W.D. (1969) *Nature*, 224, 240-243.
111. Träuble, H. (1971) *J. Membrane Biol.*, 4, 193-208.
112. Johnson, S.M. (1973) *Biochim. Biophys. Acta*, 307, 27-41.
113. Montgomery, R., and Severson, C.A. (1969) *Quantitative Problems in the Biochemical Sciences*, p. 200. W.H. Freeman - San Francisco.
114. de Bernard, L., and Dervichian, D.G. (1955) *Bull. Soc. Chim. Biol.*, 37, 943-955.
115. Demel, R.A., van Deenen, L.L.M., and Pethica, B.A. (1967) *Biochim. Biophys. Acta*, 135, 11-19.
116. Joos, P., and Demel, R.A. (1969) *Biochim. Biophys. Acta*, 183, 447-457.
117. Forge, A., Knowles, P.F., and Marsh, D. (1978) *J. Membrane Biol.*, 41, 249-263.
118. De Kruyff, B., de Greef, W.J., van Eyke, R.V.W., Demel, R.A., and van Deenen, L.L.M. (1973) *Biochim. Biophys. Acta*, 298, 479-499.

119. Singer, M.A., and Wan, J.K. (1975) *Can. J. Physiol. Pharmacol.*, 53, 1065-1071.
120. Butler, K.W., and Smith, I.C.P. (1978) *Can. J. Biochem.*, 56, 117-122.
121. Johannson, G. (1971) *Eur. J. Biochem.*, 21, 68-79.
122. Conney, A.H. (1967) *Pharmacological Reviews*, 19, 317-366.
123. Sladek, N.E., and Mannering, G.J. (1966) *Fed. Proc.*, 25, 418 (1290).
124. Rubin, A., Tephly, T.R., and Mannering, G.J. (1964) *Biochim. Pharmacol.*, 13, 1007-1016.
125. Orrenius, S., and Ernster, L. (1964) *Biochim. Biophys. Res. Commun.*, 16, 60-65.
126. Haugen, D.A., Coon, M.J. and Nebert, D.W. (1976) *J. Biol. Chem.*, 251, 1817-1827.
127. Myant, N.B., and Mitropoulos, K.A. (1977) *J. Lipid Res.*, 18, 135-153.
128. Van Cantfort, J., and Gielen, J. (1975) *Eur. J. Biochem.*, 55, 33-40.
129. Mitropoulos, K.A., and Balasubramaniam, S. (1971) *Biochem. J.*, 128, 1-9.
130. Brown, M.J. (1972) Ph.D. Thesis, University of Edinburgh.
131. Van Lies, J.E., and Smith, L.L. (1968) *Anal. Biochem.*, 24, 419-430.
132. Mitropoulos, K.A., Balasubramaniam, S., and Myant, N.B. (1973) *Biochim. Biophys. Acta*, 326, 428-438.
133. Bjorkhem, I., and Danielsson, H. (1974) *Anal. Biochem.*, 54, 508-516.
134. Saba, N., Hechter, O., and Stom, D. (1954) *J. Am. Chem. Soc.*, 76, 3862-3864.

135. Simpson, E.R., and Mason, J.I. (1976) *Pharmacol. Ther. B.*, 2, 339-369.
136. Hume, R. (1978) Ph.D. Thesis, University of Edinburgh.
137. Simpson, E.R., Jefcoate, C.R., and Boyd, G.S. (1971) *FEBS Lett.*, 15, 53-58.
138. Kovitz, S.B., and Kumar, A.M. (1970) *J. Biol. Chem.*, 245, 152-159.
139. Johnson, R.L., Ruhmann-Wennhold, A., and Nelson, O.H. (1973) *Ann. N.Y. Acad. Sci.*, 212, 307-318.
140. J.I. Mason, personal communication.
141. Arthur, J.R., Blair, H.A.F., Boyd, G.S., Mason, J.I., and Suckling, K.E. (1976) *Biochem. J.*, 158, 47-51.
142. Arthur, J.R., Mason, J.I., and Boyd, G.S. (1976) *FEBS Lett.*, 66, 206-209.
143. Simpson, E.R., and Williams-Smith, D.L. (1975) *Biochim. Biophys. Acta*, 404, 309-320.
144. Klappauf, E. personal communication.
145. Lu, A.Y.H., Junk, K.W., and Coon, M.J. (1969) *J. Biol. Chem.*, 244, 3714-3721.
146. Bruckdorfer, K.R., Edwards, P.A., and Green, C. (1968) *Eur. J. Biochem.*, 4, 506-511.
147. Shinitsky, M., and Inbar, M. (1974) *J. Mol. Biol.*, 85, 603-615.
148. Shattil, S.J., Galindo, R.A., Bennet, J., Calman, R.W., and Cooper, R.A. (1975) *J. Clin. Invest.*, 55, 636-643.
149. Graham, J.M., and Green, C. (1967) *Biochem. J.*, 103, 16C.
150. Poznansky, M.J., and Lange, Y. (1978) *Biochem. Biophys. Acta*, 506, 256-264.
151. Bloj, B., and Zilversmit, D.B. (1977) *Biochemistry*, 16, 3943-3948.
152. Poznansky, M.J., and Lange, Y. (1976) *Nature*, 259, 420-421.

153. Smith, R.J.M., and Green, C. (1974) FEBS Lett., 42, 108-111.
154. Bruckdorfer, K.R., Demel, R.A., de Gier, J., and van Deenen, L.L.M.  
(1969) Biochim. Biophys. Acta, 183, 334-345.
155. Lange, Y., and D'Alessandro, J.S. (1977) Biochemistry, 16,  
4339-4343.
156. Stevens, R.W., and Green, C. (1972) FEBS Lett., 27, 145-148.
157. Horwitz, C., Kout, L., and Kaminsky, L. (1971) Biochim. Biophys.  
Acta, 239, 329-336.
158. Green, J.R., Edwards, P.A., and Green, C. (1973) Biochem. J.,  
135, 63-71.
159. Hauser, H.O. (1971) Biochim. Biophys. Res. Commun., 45, 1049-1055.
160. Kahane, I., and Razin, S. (1977) Biochim. Biophys. Acta, 471,  
32-38.
161. Zilversmit, D.B., and Hughes, M.E. (1976) Methods in Membrane  
Biology, 7, 211-238.
162. Suckling, K.E. personal communication.
163. Mitropoulos, K.A., Venkatesan, S., Balasubramaniam, S., and  
Peters, T.J. (1978) Eur. J. Biochem., 82, 419-429.
164. Balasubramaniam, S., Mitropoulos, K.A., and Myant, N.B.  
(1973) Eur. J. Biochem., 34, 77-83.
165. Shapiro, D.J., and Rodwell, V.W. (1971) J. Biol. Chem., 246,  
3210-3216.
166. Gustaffsson, B.E., Angelin, B., Einarsson, K., and Gustafsson, J.A.  
(1977) J. Lipid Res., 18, 717-721.
167. Chevallier, F. (1978) Biochem. Soc. Trans., 6 (in press).
168. Mayer, D. (1972) "Bile Acids in Human Diseases II",  
(Back, P. and Gerok, W., eds.) p. 105-109. F.K. Schattauer  
Verlag - Stuttgart.
169. Astruc, M., Tabacik, C., Descomps, B., and Crostis de Paulet, A.  
(1974) FEBS Lett., 47, 66-71.

170. Avignan, J., Goodman, D.S., and Steinberg, D. (1963) *J. Biol. Chem.*, 238, 1283-1286.
171. Johnson, R.C., and Shah, S.N. (1973) *Biochem. Biophys. Res. Commun.*, 53, 105-111.
172. Gibbons, G.F. (1974) *Biochem. J.*, 144, 59-68.
173. Scallen, T.J., Schuster, M.W., and Dhar, A.K. (1971) *J. Biol. Chem.*, 246, 224-230.
174. Ritter, M.C., and Dempsey, M.E. (1971) *J. Biol. Chem.*, 246, 1536-1539.
175. Ritter, M.C., and Dempsey, M.E. (1973) *Proc. Natl. Acad. Sci. U.S.*, 70, 265-269.
176. Deluca, H.F. (1971) *Rec. Prog. Hormone Res.*, 27, 479-516.
177. Grabowski, G.A., McCoy, K.E., Williams, G.C., Dempsey, M.E., and Hanson, R.F. (1976) *Biochim. Biophys. Acta*, 441, 380-390.
178. Scallen, T.J., Seetharam, B., Srekantaiah, M.V., Hansbury, E., and Lewis, M.K. (1975) *Life Sci.*, 16, 853-874.
179. Saat, Y.A., and Block, K.E. (1976) *J. Biol. Chem.*, 251, 5155-5160.
180. Scholan, N. (1969) Ph.D. Thesis, University of Edinburgh.
181. Gaylor, J.L., and Delwiche, C.V. (1976) *J. Biol. Chem.*, 251, 6638-6645.
182. Ericksson, S.K., Meyer, D.J., and Gould, R.G. (1978) *J. Biol. Chem.*, 253, 1817-1826.
183. Mester, J., Robertson, D.M., Feherty, P.A., and Kellie, A.E. (1970) *Biochem. J.*, 120, 831-836.
184. Hattersley, N.G.H. (1975) Ph.D. Thesis, University of Edinburgh.
185. Mitropoulos, K.A., Balasubramaniam, S., Venketisan, S., and Reeves, B.E.A. (1978) *Biochim. Biophys. Acta*, 530, 99-111.
186. Boyd, G.S., Grimwade, A.M., and Lawson, M.E. (1973) *Eur. J. Biochem.*, 37, 334-340.

187. van der Vusse, G.J., Kalkman, M.L., van Winsen, M.P.I., and van der Molen, H.J. (1976) *Biochim. Biophys. Acta*, 428, 420-431.
188. Hauser, H., Hinckley, C.C., Krebs, J., Levine, B.A., Phillips, M.C., and Williams, R.J.P. (1977) *Biochim. Biophys. Acta*, 408, 364-377.
189. De Pierre, J.W., and Ernster, L. (1977) *Ann. Rev. Biochem.*, 46, 201-262.
190. Galla, H.J., and Sackmann, E. (1975) *Biochim. Biophys. Acta*, 401, 509-529.
191. Engelman, D.M., and Rothman, J.E. (1972) *J. Biol. Chem.*, 247, 3694-3697.
192. Hope, M.J., Bruckdorfer, K.R., Hart, C.A., and Lucy, J.A. (1977) *Biochem. J.*, 166, 255-263.



## BBA Report

---

BBA 71334

### OPTIMUM INTERACTION OF STEROL SIDE CHAINS WITH PHOSPHATIDYLCHOLINE

IAIN F. CRAIG, GEORGE S. BOYD and KEITH E. SUCKLING

*Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG (U.K.)*

(Received September 27th, 1977)

#### Summary

The specificity of the interaction between the cholesterol side chain and egg phosphatidylcholine was precisely defined by examining the effect of three new analogues of cholesterol with modified side chains on the ordering of two steroid spin labels in liposomes. The complete side chain of cholesterol was shown to be required for maximum ordering. Sterols with side chains shorter or longer than cholesterol caused significantly less ordering.

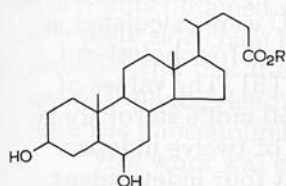
---

The role of cholesterol in membrane structure has been examined by a variety of physical techniques including NMR and ESR (for example, see refs. 1,2). The results of these and other studies are consistent with the interpretation that cholesterol causes the membrane phospholipid to possess an intermediate fluidity between a gel and a fluid state. Many different studies have shown that for maximum interaction with a phospholipid the sterol requires a planar ring structure, an equatorial  $3\beta$ -hydroxyl group and an intact side chain [3].

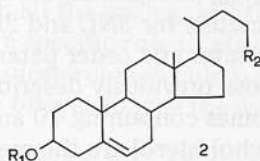
The role played by the side chain in this interaction has been studied in several ways [3–5]. Stevens and Green [4] measured the incorporation of several testosterone esters into egg phosphatidylcholine liposomes. The optimal incorporation was observed when the fatty acyl moiety had eight carbons, corresponding approximately to the length of the side chain of cholesterol. However, testosterone esters are not closely analogous to cholesterol in their molecular shape and polarity. In this laboratory we have examined the effect, by spin labelling, of varying the side chain lengths with sterols containing the cholesterol ring system [5]. These analogues are more closely related to cholesterol, the only difference being the modified side chains. This work suggested that sterols with side chains shorter than cholesterol by more than three carbons caused significantly less ordering of the spin labels in egg phos-

phatidylcholine liposomes than did cholesterol. These results encouraged us to synthesise further analogues of cholesterol to define the requirement for a side chain in the interaction with phospholipid more precisely. In this paper we report on the synthesis of three new analogues of cholesterol (Fig. 1) and their effect in egg phosphatidylcholine liposomes on the ordering of the two steroid spin labels described before, 3-spiro(2'-(*N*-oxyl-4',4'-dimethyloxazolidine))cholestane (3NC) and  $\beta$ -hydroxy-26-nor-25 (2'-(*N*-oxyl-4',4'-dimethyloxazolidine))cholestane (25NC) [5].

Reagents used were of standard commercial grade and purified as required. Egg yolk phosphatidylcholine was prepared using the method of Pangborn [6] and was pure by thin-layer chromatography. Liposomes were prepared containing sterols, phospholipid and spin labels and ESR spectra obtained as described previously [5].



1a R = H  
1b R = Me



2	R <sub>1</sub>	R <sub>2</sub>
a	H	— CO <sub>2</sub> H
b	H	— CO <sub>2</sub> Me
c	Tetrahydropyranyl	— CO <sub>2</sub> Me
d	"	— CH <sub>2</sub> OH
e	"	— CHO
f	"	— CHOHCH <sub>3</sub>
g	"	— CHOHCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>
h	"	— CHOH(CH <sub>2</sub> ) <sub>4</sub> CH <sub>3</sub>
i	H	— CH <sub>2</sub> CH <sub>3</sub>
j	H	— (CH <sub>2</sub> ) <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>
k	H	— (CH <sub>2</sub> ) <sub>5</sub> CH <sub>3</sub>

### Synthesis of cholesterol analogues

Hydoxycholelic acid (1a) was esterified with diazomethane and the ester (1b) converted into  $\beta$ -hydroxycholelic acid (2a) by the method of Bharuca et al. [7]. After esterification with diazomethane the  $\beta$ -hydroxycholelic acid methyl ester (2b) was converted into its tetrahydropyranyl ether (2c) [8]. The ether was reduced to the corresponding alcohol (2d) with lithium aluminium hydride and the alcohol oxidised to the protected aldehyde (2e) with celite-silver oxide [9]. The aldehyde was then reacted with the appropriate Grignard reagent [10] and the resulting secondary alcohol (2f, 2g, 2h) reduced via its tosylate to give a saturated side chain essentially as described by Arthur et al. [11]. Deprotection was effected by treatment of the steroid with acid [8] and the product was purified by chromatography and recrystallisation. All intermediates were characterised by infrared and NMR

spectroscopy and mass spectrometry and were shown to be pure by thin-layer chromatography. The product sterols were shown to be pure by thin-layer chromatography and by gas liquid chromatography. Their physical characteristics were as follows:

24-methyl-5-cholesterol, m.p. 133°C,  $M^+$  358  $C_{25}$  sterol (2i);

26-nor-27,27-dimethyl-5-cholesten-3 $\beta$ -ol, m.p. 128–129°C,  $M^+$  400  $C_{28}$  sterol (2j);

26-nor-27-*n*-propyl-5-cholesten-3 $\beta$ -ol, m.p. 124–125°C,  $M^+$  414  $C_{29}$  sterol (2k).

#### Order parameters for 3NC and 25NC

Experiments were carried out using the  $C_{25}$ ,  $C_{28}$ , and  $C_{29}$  analogues and also with cholesterol using egg phosphatidylcholine liposomes containing 0–50 mol% sterol. Order parameters for 3NC and 25NC were calculated as described previously and the spectra and order parameters for cholesterol were essentially the same as those previously described [5]. The values of the order parameters for liposomes containing 40 and 50 mol% sterol are given in Table I. The results for cholesterol are the mean of twelve independent experiments and those for the analogues of at least four independent

TABLE I

ORDER PARAMETERS FOR CHOLESTEROL ANALOGUES IN EGG PHOSPHATIDYLCHOLINE LIPOSOMES

mol% sterol		Sterol			
		$C_{27}$	$C_{25}$	$C_{28}$	$C_{29}$
3NC	40	0.34 $\pm$ 0.02	0.30 $\pm$ 0.01	0.31 $\pm$ 0.01	0.31 $\pm$ 0.01
	50	0.39 $\pm$ 0.02	0.35 $\pm$ 0.01	0.36 $\pm$ 0.01	0.36 $\pm$ 0.01
25NC	40	0.264 $\pm$ 0.01	0.255 $\pm$ 0.01	0.257 $\pm$ 0.01	0.255 $\pm$ 0.01
	50	0.29 $\pm$ 0.01	0.270 $\pm$ 0.01	0.270 $\pm$ 0.01	0.270 $\pm$ 0.01

experiments. In every case the order parameter was found to increase with increasing sterol concentration. The presence of cholesterol caused significantly more ordering than either the shorter ( $C_{25}$ ) or the longer ( $C_{28}$ ,  $C_{29}$ ) side chain analogues at concentrations greater than 40 mol% with both spin labels. The differences in order parameter at compositions below 40 mol% were too small to be distinguishable by these methods.

These results and those reported previously agree in outline with those of Stevens and Green [4]. They found that a testosterone ester with an eight carbon side chain gave maximum incorporation into egg phosphatidylcholine liposomes. They suggested that since this compound is slightly longer than cholesterol, a cholesterol-like molecule with one extra carbon in the side chain (i.e., our  $C_{28}$  analogue) would be incorporated to a greater extent than cholesterol. Although our experimental conditions are not directly comparable, the spin label results indicate that this may not be the case. Cholesterol

itself appears to have the optimal structure for a maximum sterol-phospholipid interaction.

Recent results from X-ray and neutron diffraction studies [12,13] show that cholesterol occupies a position in a phosphatidylcholine bilayer such that its side chain terminates in the region of the ends of the fatty acyl chains of the phospholipid. On this basis, the C<sub>28</sub> analogue and more especially the C<sub>29</sub> analogue, which is much longer, would be likely to penetrate into the terminal region of the adjacent half of the phospholipid bilayer. Such an interaction or the change that might take place in a phospholipid bilayer to relieve the consequences of such an interaction would be likely to result in a decrease in order of the bilayer. This is what is observed in these experiments. The order of the liposomes containing the sterols with longer side chains is reduced to the same level as that observed with sterols with shorter side chains.

These results confirm and extend our previous findings. Taken together, the results obtained with all the analogues of cholesterol suggest that the complete side chain has a specific role to play in the interaction with phospholipid and that the cholesterol molecule is the optimum size to fit into an egg phosphatidylcholine bilayer to give the maximum possible interaction with the phospholipid.

This work was supported by the Medical Research Council. I.F.C. acknowledges the award of a MRC Research Studentship.

## References

- 1 Opella, S.J., Yesimowski, J.P. and Waugh, J.S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 3812-3815
- 2 Shimshick, E.J. and McConnell, H.M. (1973) *Biochem. Biophys. Res. Commun.* 53, 446-451
- 3 Demel, R.A. and de Kruijff, B. (1976) *Biochim. Biophys. Acta* 457, 109-132
- 4 Stevens, R.W. and Green, C. (1972) *FEBS Lett.* 27, 145-148
- 5 Suckling, K.E. and Boyd, G.S. (1976) *Biochim. Biophys. Acta* 436, 295-300
- 6 Pangborn, M.C. (1951) *J. Biol. Chem.* 188, 471-476
- 7 Bharuca, K.R., Buckley, G.C., Ross, C.K., Rubin, L.J. and Ziegler, P. (1956) *Can. J. Chem.* 34, 982-990
- 8 Petrov, V. and Stuart-Webb, I.A. (1956) *J. Chem. Soc.* 4675-4677
- 9 Fried, J. and Edwards, J.A. (1972) *Organic Reactions in Steroid Biochemistry*, van Nostrand Reinhold, New York
- 10 Dasgupta, S.K., Crump, D.R. and Gut, M. (1974) *J. Org. Chem.* 39, 1658-1662
- 11 Arthur, J.R., Blair, H.A.F., Boyd, G.S., Mason, J.I. and Suckling, K.E. (1976) *Biochem. J.* 158, 47-51
- 12 Franks, N.P. (1976) *J. Mol. Biol.* 100, 345-358
- 13 Worcester, D.L. and Franks, N.P. (1976) *J. Mol. Biol.* 100, 359-378